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The Persistence and Regularity of Electrical Activity in Whole Heart Explants

By

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Abstract

CUNNINGHAM, A. W. B., N. O. LUNELL and B. J. RYLANDER. *The persistence and regularity of electrical activity in whole heart explants.* Acta physiol. scand. 1961. *52.* 101—107. — The electrical persistence and regularity of 143 chick embryo whole heart explants during nine days in culture is described. Details are given of culturing and surveillance of explants and handling of data. Over 90% of explants showed continuous electrical activity for nearly all of the first three days in culture. This percentage fell 6 % by the end of day three, 6 % during day four, 17 % on day five and more slowly thereafter. These changes possibly reflected inadequacies in the supernatant. The frequency and magnitude of change in the number of active cultures from one 15 second survey to the next slowly increased with a maximum of 1.2 cultures. It seemed that the failure of hearts to produce potentials was usually temporary and occurred in a rhythmic fashion. The difference between consecutive half-hour averages of the number of cultures showing activity during 15 out of every 20 seconds was relatively high initially, dropped during the second day then rose again. It was always small compared to the total number of cultures involved. Comparisons between the persistence of electrical function in whole heart explants in various supernatants showed that the one used in this study was least inadequate.

The contractile activity of chick embryo hearts has been previously followed by microscopic observation and recording (v. BONSDORFF 1951). These observations were made intermittently since no automatic recording technique was used. In the present investigation, observations were made on 143 whole

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chick embryo hearts for up to 216 hours after explantation using a more continuous automatic recording technique. The change in the number of hearts showing electrical activity as a result of the passage of time was measured.

Techniques

The 143 hearts were observed in 4 groups (the incubators held a maximum of 40 cultures): (A), 38 hearts observed from 124—215 hours after explantation, (B), 33 hearts observed from 4 1/2—230 hours after explantation, (C), 36 hearts observed from 3 1/2—145 hours after explantation and (D), 36 hearts observed from 4 1/2—150 hours after explantation.

Each heart was explanted from a 7-day embryo chick onto a 36 gauge, bare platinum electrode in the angle of an L-shaped piece of cellulose sponge held onto a coverglass by Diatex (CUNNINGHAM and LUNELL 1960). Diatex is a toluol solution of a modified acrylic acid compound which, when properly dried, gives a hard transparent film adherent to glass and, in our experience, non-toxic to heart cultures. Each explant on its electrode and coverglass was placed in a separate culture chamber equipped with 36 gauge platinum reference electrode and a flow system giving a uniform flow of 1 ml per day (CUNNINGHAM and LUNELL 1960). The culture chamber and flow system were clipped onto a metal frame on which the electrodes were connected, by insulated connectors, to a shielded cable. The whole system was kept in an incubator at 37° C.
Supernatant; — This was a 0.25 % solution of human serum protein in the following balanced salt solution; (Tissue Dynamics Laboratory Balanced Salt Solution, TDLI).

	g/l		meq/l
Potassium Chloride	0.28	Potassium	4.15
Sodium Chloride	7.0	Sodium	134.0
Calcium Chloride	0.28	Calcium	5.04
Magnesium Sulphate	0.15	Magnesium	2.44
Disodium Phosphate	0.08	Phosphates	1.44
Monopotassium Phosphate	0.04		
Sodium Bicarbonate	1.05	Bicarbonate	12.5
Sodium Acetate	0.05	Sulphate	2.44
Dextrose	1.75	Chlorides	128.5
Water to one liter			

Collection of Data

All heart explants in the study were surveyed automatically using an electronic instrument which detected and recorded the total number of hearts giving rise to a minimum of one 0.05 mV potential during the first 15 out every 20 sec. Potentials arising in any of the hearts were amplified and fed into a channel in the electronic recording apparatus (there was a separate channel for each heart under surveillance) where the first impulse of more than 0.05 mV to arrive, activated an electronic switch which then remained activated for the whole 15 sec survey. At the end of the 15 sec survey, a measurement was made of the total amount of current flowing through all the activated switches. This value was registered on a stripchart recorder as a straight line (Fig. 1) and its length was directly proportional to the number of heart explants which had given rise to one or more potentials greater than 0.05 mV during the past 15 sec. Following the 15 sec survey there was a 5 sec silence to allow the detecting mechanisms and recording pen to reset.

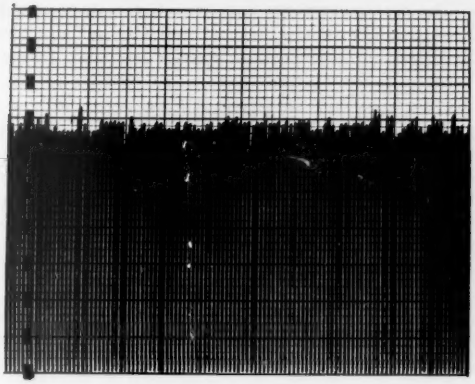

Fig. 1. A half hour strip of the read-out from the automatic electronic monitor. Each peak represents the number of explants which have given rise to one or more potentials of greater that 0.05 mV during the previous 15 seconds. Each horizontal line represents one explant.

Processing of Data

Using the above apparatus, over two million observations were made on 143 cultures in over one hundred thousand 15 sec surveys. A sequence of values representing the averages of all 15 sec surveys for each half-hour were obtained by planimetry. This was done by measurement of the area between the baseline and a line joining the peaks of the lines representing the values obtained from each 15 sec survey during that half-hour. To facilitate comparison of the results from different groups of cultures, the planimetric average for each half hour was expressed as a percentage of the planimetric average for a half hour during which it was judged that every culture in that particular group showed electrical activity every 15 sec.

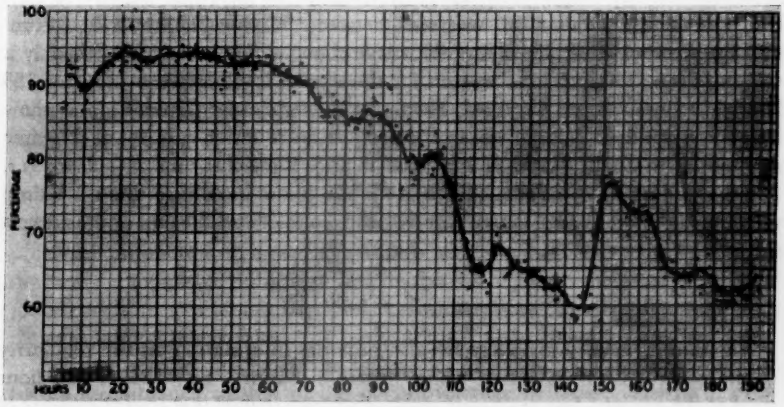

Fig. 2. Y axis — Half hour averages of the number of cultures showing electrical activity during 15 out of every 20 seconds (consecutively), expressed as a percentage of the total number of explants showing activity at the beginning of the experiment. From the time of explantation till 145 hours Groups B, C and D were involved, from 145—215 hours Groups A and B were involved and from 215—240 hours only Group B was involved. Dotted line-actual half hour averages, Solid line — 5 hour running average. X axis — Time in hours.

The processing of the data from Group A provided a special difficulty since the planimetric values for a half-hours "full activity" in all cultures in this group (which would have been the 100 % value) was not available. Therefore, in order to assign a percentage value to each half-hour planimetric average in this group, the mean of all half hour planimetric values for Group A from 144—168 hours after explantation was arbitrarily given, as a percentage value, the mean of all the percentage persistence values for the period 144—168 hours in culture from Group B. Thus a relationship between half-hour averages of the 15 sec surveys and percentage electrical persistence was established and from it percentage values could be worked out for each planimetric value for Group A. Also, since the percentage values for Group B between 144—168 hours in culture were higher than those likely to have been found in Group C or D, the percentage values for Group A were also higher. Hence the curves representing the survival of electrical potential production ran at a higher level for the period after 150 hours in culture (when it is constructed from data from Groups A and B) than before 150 hours in culture (when it is constructed from data from Groups B, C and D) (Fig. 2).

Results

Data on Persistence of Electrical Potential Production

The curve constructed from the average of the half-hour planimetric values for the four groups of cultures (B, C, and D before 150 hours and A and B after 150 hours) is given in Fig. 2. The heavy line which represents the 5 hour running average, shows that there was an increase of 3 % in the number of functioning cultures during the first 24 hours in culture. During almost all of the next 45 hours, the average percentage of active cultures remained over the 90 % level but had a drop of 4 %. The period from 69—92 hours in culture showed a slightly longer drop in the persistence of electrical activity (6 %), while the sharpest fall of all was seen between 92 and 117 hours in culture (20 %). From 118 till 144 hours the curve shows a flattening out with only a 4 $^1/_2$ % drop. The principal reason for the rise in level between 145 and 153 hours has already been discussed. However, the figures for Group B alone, show that there is indeed a slight rise in the proportion of active cultures at this time amounting to about 4/5ths of that seen in Fig. 2 for this time in culture (*i.e.* 8 % instead of 10 %). From this time till 176 hours in culture there is a drop of 10 % in the number of active cultures and from 176 till the end of the curve a drop of 5 %.

Data on Regularity of Electrical Behavior

The data available in this study also provides information on the regularity of the contractile behavior of whole hearts in culture. Fig. 1. is a half hour strip of the straight-line read-outs, each of which represents the total number of hearts producing a potential of more than 0.05 mV during a 15 sec survey period. This strip was taken at random from those for this study and shows the remarkably uniform behavior of the hearts in this respect. An examination of a similar strip of recording taken for the period 8.30—9.00 p. m. during each day for

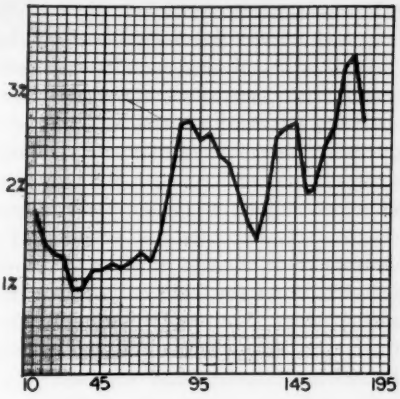

Fig. 3. Y axis — The five hour running average of the change (increase or decrease) in the number of explants showing electrical activity during a 15 second surveillance. This running average was constructed from the combined half hour averages from all four groups and corrected to represent a percentage of the total number of hearts actually showing activity at the time of the change.
X axis — Time in hours.

each of the four groups of cultures shows that the number of cultures showing electrical activity per fifteen second survey, varies to a relatively great extent during the first 24 hours. During this time there is an average change of 0.45 in the number of active cultures, between successive 15 sec surveys during the half hour. During the next 48 hours this value dropped to 0.3 cultures then rose again till by 144 hours it was about 0.5 cultures. However, for every increase in the number of electrically active cultures, there was a subsequent and almost equal decrease so that the ultimate net change in the number of cultures was not large.

Fig. 3 shows the 5 hour running average of the change (increase and decrease) in the number of electrically active cultures.

The running average was constructed from the half-hour averages for all four groups of cultures. It is corrected to represent a proportion of the total number of hearts actually showing electrical activity at the time of the measurement of the change.

Discussion

Our present knowledge of the factors governing the survival of electrical potential production in heart explants is small and we have been unable to find truly comparable published data on this problem, save in papers previously published from this laboratory. Hence discussion is necessarily limited and the paper is largely descriptive.

The form of the curves for the individual groups of cultures had a definite family resemblance to that of the overall average behavior. The form of the curve in Fig. 2 is probably the result of a combination of different causes of myocardial damage such as;

a) the effects of the products of mechanical tissue damage inflicted during explantation,

b) malnutrition from poor physical distribution of food and oxygen,

c) lack of essential materials originally in short supply,

d) diffusion of vital metabolic intermediaries through damaged cell membranes.

In this curve, the initial 3 % rise during the first 24 hours was probably due to recovery from the trauma of culturing. The 45 hour plateau above 90 % electrical persistence which follows suggests that, during this time, there was a sufficient supply of vital metabolic materials. Then the sharp drop which progressively becomes less, presumably reflects inadequcy in the artifical environment. While the nutritional demand from a relatively large number of active cultures was high early in this phase, there was a sharp decline in the ability of some cultures to maintain electrical persistence. Later in this phase there were fewer active cultures and presumably lesser nutritional demand hence the decline became less.

Some further comments on this curve will be found in other parts of this discussion.

The Regularity of Electrical Activity in Cultures

The method used to record activity in this study was such that a change in the number of active cultures may mean that a culture was;

a) starting for the first time,

b) stopping finally,

c) beating irregularly so that it was constantly making reentries,

d) beating regularly less than once per 15 sec so that it did not appear in every 15 sec scan.

The number of electrically active cultures per 15 sec survey shows a trend of increased variation after 72 hours even when a daily sample of only one half-hours activity is examined. The level of variation is small when compared with the total number of cultures involved. Also, the total number of cultures showing electrical activity per survey period increases nearly as often as it decreases. This suggests that a culture usually lost electrical activity only for a short time. The progressive increase in the amount of variation in the number of active cultures as they aged is seen in Fig. 3. A comparison of Fig. 2 and 3 shows that the greatest change in the number of active cultures co-incided with the greatest fall in the total number of active cultures. Thus, in general there is a progressive increase in the frequency and magnitude of irregularity in the number of electrically active cultures as they aged.

The Effect of Electrolytes on Electrical Persistence

The effects of the electrolyte composition of the supernatant on the persistence of electrical activity in the cultures can be measured by a comparison between the data from this study and that from previous similar studies in this laboratory. A previous paper (Estborn *et al.* 1958) gave information on the

electrical persistence in whole heart explants cultured in synthetic supernatant 199. The criteria for a 192 hour survival (the production of a 0.02 mv potential during one of 29, $7^1/_2$ second surveys between 192 and 216 hours in culture) would probably be regarded as a 216 hour survival in the present study. Using synthetic supernatant 199 only 61 out of 258 hearts (24 %) showed adequate electrical function for 215 hours in culture. In the present study more than 50 % cultures showed electrical activity at 216 hours in culture. Thus, our present supernatant permitted twice as high an electrical persistence in whole hearts in culture than did synthetic supernatant 199.

Another previous study (CUNNINGHAM, KLEUTCH and HERBST 1960) gave a comparison of the persistence of electrical activity in supernatants of differing potassium levels. It concluded that, of those tried, the supernatants with a potassium level of 4.0 meq/l gave the best result. Our present supernatant has a potassium level of 4.15 meq/l and its levels of sodium, magnesium, calcium and phosphate are more physiological. The proportion of cultures showing electrical persistence at 168 hours in the 4.0 meq/l was 52 %. In the present study it is between 62 and 73 %. Thus the regulation of all of the electrolytes to physiological levels causes a 20 % greater electrical persistence at 168 hours in culture.

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The Discharge in Single Touch Receptors Elicited by Defined Mechanical Stimuli

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Abstract

Höglund, G. and U. Lindblom. *The discharge in single touch receptors elicited by defined mechanical stimuli.* Acta physiol. scand. 1961. *52.* 108—119. — To understand how a touch stimulus is coded in nerve impulses it is fundamental to know the significance of its wave form. This has been studied in the toad by means of a special technique for stimulation of individual touch receptors in situ with simultaneous recording of the skin displacement. Stimuli of various gradients, amplitudes and durations were applied to the skin surface over the receptor, and the impulse discharge in the afferent fibre was recorded from thin filaments of the dorsal root.

The threshold amplitude was remarkably independent of the stimulus gradient. When the latter was increased gradually from the minimum effective value (critical slope) and upwards, the threshold fell sharply at first but then remained at the same level (Fig. 3 and 4).

Prolongation of a threshold stimulus by adding a plateau phase to the initial moving phase did not result in a repetition of the response and did not make it possible to reduce the amplitude (Fig. 1). The repetitive response usually obtained on suprathreshold stimulation was either limited to the moving phase or had the highest impulse frequency during this phase (Fig. 2).

Since the strongest excitation occurred during the moving phase of the stimuli, it was concluded that the deformation is the adequate stimulus. The deformed state is normally followed by no response at all or, in some receptors, by a rapidly adapting discharge.

In a recent paper the excitability of touch receptors of the type that is connected to coarse afferent fibres was studied in situ on the toad (Lindblom 1958). These receptors were shown to have a relatively refractory period of a con-

siderable duration, and the refractoriness left behind by the individual impulses was found to summate during repetitive firing so as to counteract further discharge from the receptor. It was considered to be of interest to investigate quantitatively in the same type of preparation other factors that influence the impulse frequency and the duration of the discharge. Some of these factors are the excitability properties of the receptors while others belong to the stimulus. The present paper describes the effect on the discharge of varying the form of a defined mechanical stimulus. A preliminary report has been given at the X Scandinavian Congress in Physiology in Oslo (HÖGLUND and LINDBLOM 1960).

There are several earlier observations that concern the relation between the characteristics of the stimulus and the discharge in touch receptors of the type supplied by coarse afferent fibres. As was originally shown by ADRIAN and ZOTTERMAN (1926) in the cat, the typical response in the coarse afferent fibres from the skin on application of slight pressure is a short-lasting high-frequency burst of impulses. ADRIAN, CATTELL and HOAGLAND (1931) found that the discharge of the touch receptors, studied in nerve-skin preparations from the frog, could be reduced to a few impulses if the stimulus, which consisted of an air current, was applied suddenly. They assumed that impulses are set up only during the actual period of movement of the skin. The same authors also pointed out that a certain abruptness is essential to stimulation, and later GRAY and MALCOLM (1951) made direct measurements of the critical slope for discharge in the same type of preparation. For stimulation they used a piezo-electric crystal which was placed on the inside of the skin so as to stimulate the termination of a single axon.

The advantages with the technique we have used, apart from the receptors being studied in situ with intact circulation, are that the adequate stimulus can be localized to a single touch-sensitive point in the receptive field and defined in terms of displacement of the skin surface. The type of preparation used has not allowed a direct study of local potentials in the receptor region or the accommodative properties of the fibre endings. This is a clear limitation from a theoretical point of view. For the present purpose, however, the essential thing is that the message in the afferent fibres could be related quantitatively to the physical characteristics of the stimulus. In order to obtain skin displacements of variable slope, amplitude and duration a specially designed mechanical stimulator has been used (HAAPANEN 1960).

Methods

All experiments were performed on decerebrate toads (*Bufo bufo*). 101 receptors belonging to 65 tactile units (see LINDBLOM 1958) with their receptive fields on the lower leg or plantar surface of the foot were studied. Action potentials were led off from single afferent fibres in the dorsal root. The stimulator (HAAPANEN 1960) gave linearly rising mechanical pulses, the rate of rise of which was continuously variable between 0.8 and 200 mm/sec. The movement could be interrupted at any moment during the rising

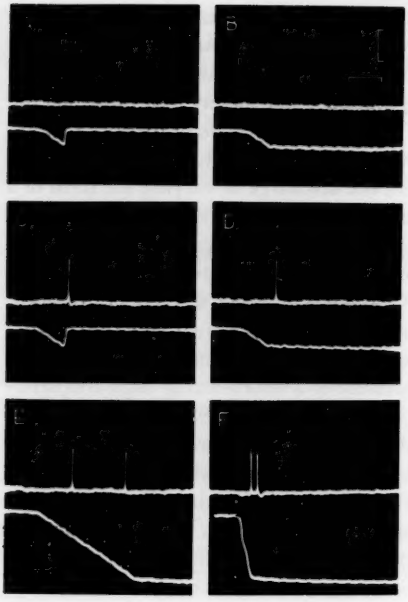

Fig. 1. Records from dorsal root fibre (upper beam) and capacitance meter (lower beam) illustrating the ineffectiveness of steady stimulation at subthreshold (A and B), threshold (D) and suprathreshold (E and F) stimulus amplitude. F shows also the effect of increasing the stimulus gradient. Horizontal bar: 50 msec. Vertical bar: 100 μ. Stimulus gradient in A—E 1.8 mm/sec, in F 13 mm/sec. From touch receptor on proximal part of plantar surface.

phase and the stimulator brought back to zero position or maintained at the level of interruption for a variable length of time so as to give a steady displacement of the skin. The maximal amplitude of movement was 260 μ. The stimuli were applied to the skin surface by means of a small blunt rod, which was adjusted so as to produce a vertical displacement of the skin overlying the receptor. Before stimulation, the stimulator, which was mounted on a micromanipulator, was put in contact with the skin without compressing it. Care was also taken not to stretch the skin when the leg was fixed prior to stimulation (cf. LOEWENSTEIN 1956 a). The displacement of the skin surface was recorded on each stimulation by means of a capacitance meter as has been described previously (LINDBLOM 1958, p. 12).

Results

On stimulation with *threshold amplitude*, the response always consisted of a single impulse. Fig. 1 C shows the response to a linearly increasing mechanical stimulus of threshold amplitude. Maintained displacement, as in Fig. 1 D, did not result in a repetitive discharge and was not accompanied by a lowering of the threshold amplitude[1]. This result shows that the moving phase is the effective part of the stimulus and that the adequate stimulus for these receptors

[1] In Fig. 1 C and D, as well in subsequent records, the impulse appears in the record *after* the end of the moving phase of the stimulus. This delay corresponds to the time for conduction of the impulse from the receptor to the site of recording in the dorsal root (cf. LINDBLOM 1958).

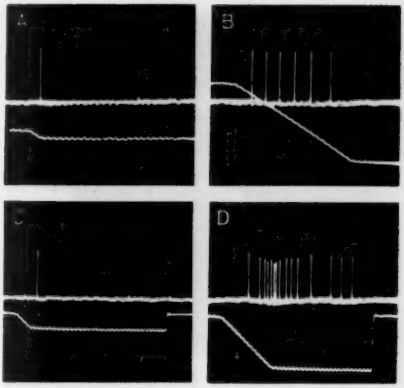

Fig. 2. Typical discharges following threshold (A and C) and suprathreshold (B and D) stimulation of two different touch receptors which both are less rapidly adapting than the receptor illustrated in Fig. 1. A and B from receptor on lateral pad of plantar surface. Horizontal bar: 100 msec. Vertical bar: 100 μ. C and D also from receptor on lateral pad but from another experiment. Horizontal bar: 100 msec. Amplitude scale same as in B. Stimulus gradient in all records 0.8 mm/sec.

is the deformation. This is further emphasized by the results of sub- and suprathreshold stimulation. In Fig. 1 A the amplitude of the stimulus is slightly less than the threshold value in C. Record B shows that no response was obtained when the displacement was maintained at the same level as in A. Thus, steady stimulation could not yield the small degree of excitation needed for discharge when it had been brought to a near-threshold level by the preceding moving part of the stimulus.

On *suprathreshold* stimulation the most rapidly adapting receptors responded with only a few impulses. For the receptor illustrated in Fig. 1, *e.g.*, an increase of the amplitude of the stimulus to 4 times the threshold value did not result in more than two impulses (record E). From other receptors a burst of several impulses could be obtained. The discharge from such a receptor is illustrated in Fig. 2 A and B, which show the response to threshold and suprathreshold stimulation, respectively. Even in this case the discharge is limited to the moving phase of the stimulus. Fig. 2 C and D show the response from a third type of receptor, which discharged also during the plateau phase of the stimulus on suprathreshold stimulation. As seen in D, the impulse frequency is highest in that part of the discharge which has been initiated during the moving phase. This type of less rapidly adapting touch receptor was the only one that responded substantially to steady stimulation. Even so, however, the discharge usually came to an end in less than a second except for single randomly discharged impulses.

The discharge in the less rapidly adapting touch receptors of the type illustrated in Fig. 2 C and D was often potentiated if the skin was displaced prior to stimulation by lowering the stimulator 100—300 μ from the ordinary zero position where the stimulator rod was just in contact with the skin surface. Under these circumstances there were two receptors whose discharge lasted a little more than half a minute on steady stimulation. For the rest of the receptors

Table I. Critical slope of 28 receptors

mm/sec.	rheob./sec.	mm/sec.	rheob./sec.
0.8–1.6		2.5– 2.6	
0.8–2.1		2.5– 3.4	
0.8–1.6		2.5– 4.2	
0.8–2.6	3.3–11	2.6	38
		2.6– 3.1	
1.0	22		
1.3–2.1		2.8	
1.4–1.6		2.8–3.0	
1.4–2.1	17–26	2.9–34.0	97–1,100(?)
1.4–3.2		3.8– 5.0	
1.9	42	3.9	
2.1–2.8	31–41	10.0–11.0	160–180
2.1–3.5	17–29	10.0–12.0	180–200
2.3–2.6		10.0–15.0	130–200
2.4–4.8		11.0–40	52–190

the potentiation consisted of a slight or moderate increase of the frequency and duration of the discharge. The sensitivity of the most rapidly adapting receptors was either uninfluenced or lowered by a preset displacement. None of the receptors was thus found to be slowly adapting in the sense that a long-lasting or stationary discharge was obtained with stimulation and recording conditions that may be considered as physiological. The material seems to be large enough to allow the conclusion that more or less rapid adaptation characterizes all low threshold mechanoreceptors in the toad's skin supplied by coarse afferent fibres.

Some receptors showed an off-response consisting of a single impulse which appeared when steady stimuli with a duration of a few hundred msec. had been applied and were sharply cut off. The occurrence of an off-response was apparently not related to the rate of adaptation or the threshold of the receptor. An after-discharge, which characterize touch receptors connected to small fibres in the cat (ZOTTERMAN 1939), or a spontaneous discharge in the absence of mechanical stimulation, was never observed.

The observations in the present investigation all show that the strongest excitation occurs during the period of deformation and that many, perhaps most, of the toad's touch receptors supplied by coarse afferent fibres do not normally discharge at all during steady stimulation irrespective of the stimulus strength.

If the stimuli to the receptor were to be effective, the rate of displacement had to be at or above a certain critical value. A lowering of the rate below this value could not be compensated for by an increase of the amplitude of the displacement. 58 of the 86 receptors which were tested for the critical slope (*c.s.*) were found to discharge on stimulation with the lowest rate of displacement

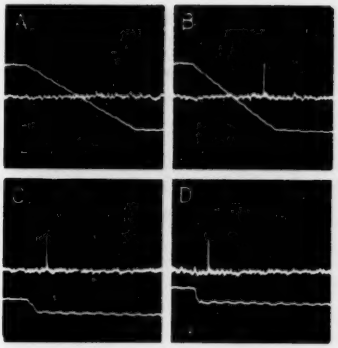

Fig. 3. Records illustrating the effect on the threshold of varying the stimulus gradient. This is in A 1.7 mm/sec., below the critical slope, in B 2.2 mm/sec, same as the critical slope, and in C and D, 4.1 and 11 mm/sec, resp. From touch receptor on proximal margin of plantar surface.

which could be obtained from the stimulator. It can therefore only be stated that the *c.s.* of these receptors was at or below 0.8 mm/sec. For the remaining 28 receptors the *c.s.* was between 0.8 and 40 mm/sec.

The *c.s.* very often showed temporal fluctuations. For some receptors identical values were obtained when the test was repeated several times, but the typical finding was that the *c.s.* varied continuously back and forth within certain limits when the same receptor was followed for some length of time. Significant variations were often found from one minute to another. As a consequence of the temporal fluctuations, the *c. s.* of a particular receptor should be given as a range rather than a single value. In table 1 the extreme values for all 28 receptors has been listed to give an idea of the magnitude of the fluctuations as well as the absolute values for the *c.s.* Some of these are also given in rheobases/sec. The threshold on stimulation with a steep gradient (corresponding to the horizontal plateau of the curve in Fig. 4) was taken as rheobasic strength. The observation time was about half an hour for each receptor.

The temporal fluctuations could not be correlated with any special circumstance in the experimental situation, such as temperature or moisture of the skin, and therefore they most probably indicate a spontaneous fluctuation in the excitability of the receptor endings. Another possibility is that they reflect centrifugal regulating activity of sympathetic fibres to the skin (LOEWENSTEIN 1956 b).

In one series of experiments the threshold amplitude was determined at various rates of displacement above the *c.s.* and the typical result is illustrated in Fig. 3 and 4. Fig. 3 A shows stimulation at the rate of 1.7 mm/sec. This stimulus was ineffective in spite of having the maximal amplitude that could be obtained from the stimulator (260 μ). When the rate of the displacement was successively increased, a propagated response was suddenly obtained at the rate of 2.2 mm/sec (Fig. 3 B). As seen, the impulse appears in the dorsal root fibre during the downward movement of the stimulator. This means that the stimulus amplitude was suprathreshold.

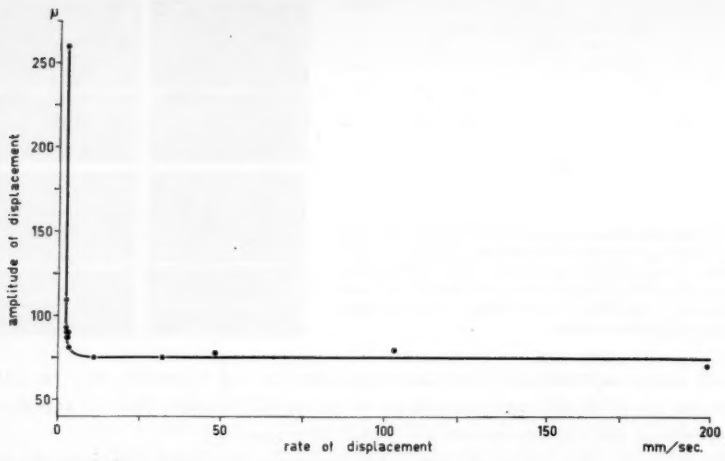

Fig. 4. Curve showing the typical relation between threshold amplitude and gradient of stimulus. From touch receptor on plantar surface. Critical slope 2.3 mm/sec.

By reducing the latency by the calculated time for conduction of the impulse from the receptor level to the site of recording in the dorsal root (cf. LINDBLOM 1958), it could be determined that the impulse in Fig. 3 B was discharged from the receptor approximately 85 msec after the beginning of the displacement of the skin surface. This means that the threshold amplitude amounted to 195 μ on stimulation at the *c.s.*

The threshold was then determined at various higher rates. This was done by reducing the amplitude of the stimulus until the response disappeared. (This method was easier to use than latency measurements but equally reliable since, as described above, the impulse is initiated during the phase of downward movement and not during the plateau phase of the stimulus). In Fig. 3 C where the rate of displacement had been increased to 4.1 mm/sec, the threshold amplitude was 55 μ, *i.e.* considerably lower than at the *c.s.* A further increase in the rate of displacement, however, did not result in a further reduction of the threshold (Fig. 3 D). The relation between the gradient and the threshold amplitude of the stimulus is shown in detail in the curve in Fig. 4, which is from another receptor. As seen, the threshold falls very rapidly at rates just above the *c.s.* and thereafter remains at the same level throughout the whole range of higher rates.

The conclusion to be drawn from these experiments is that, as soon as the gradient of the stimulus is well above the *c.s.*, the receptor is insensitive to a further rise of the gradient.

On *suprathreshold* stimulation the gradient of the stimulus is very relevant for the various features of the discharge. With stimulus rates at or near the critical

slope the latency is usually relatively long. In Fig. 3 B, *e.g.*, the receptor delay was about 85 msec. Another consequence of a low stimulus rate is that the discharge is limited by the adaptation which comes in already during the phase of deformation. This can be seen even for the receptors that are prone to fire repetitively. An increase of the gradient is followed by a shortening of the latency and an increase of the discharge frequency (Fig. 1 E and F). The extent of the frequency increase will primarily be determined by the balance between excitation and refractoriness. The latter, which summates on repetitive firing, will be more relevant than adaptation for the limitation of the discharge on stimulation with steep gradients.

Discussion

The suggestion made by ADRIAN, CATTELL and HOAGLAND (1931) that impulses in coarse tactile fibres are set up only during the period of movement of the skin has been largely confirmed in the present experiments. These have shown that the strongest excitation occurs simultaneously with the deformation of the endings while the deformed state is accompanied by a relatively weak discharge or not discharge at all. It may be pointed out that, although the technique we have used allows a quantitative evaluation of the stimulus in terms of displacement of the skin surface, the type or extent of the deformation of the mechano-sensitive structures within the skin cannot be defined. The whole tissue in which the endings are embedded is displaced and deformed by the stimulus and the deformation of them may include both compression, bending and stretch (cf. LINDBLOM 1958, Fig. 3).

During steady stimulation of touch receptors connected to coarse afferent fibres, the rapid adaptation is the most conspicuous and, from a physiological point of view, obviously the most relevant event. If the propagated response is taken as the only index of excitation this would be zero in the adapted state in which no impulses are elicited. Subliminal changes of the excitability may, however, exist in the absence of any propagated response. This was indicated in the experiments of GRAY and MALCOLM (1951) in which it was found that a conditioning stimulus of long duration might contribute up to 35 % of the threshold to a superimposed test stimulus. In the present investigation an increase in excitation was seen in the less rapidly adapting receptors in the form of an increase of the frequency and the duration of the discharge when the stimulus was repeated after adjustment of the stimulator so as to give a preset steady level of displacement. This increase in excitation was graded and paralleled the amplitude of the preset displacement. The same type of excitatory effect can be produced by horizontal stretching of the skin as shown by LOEWENSTEIN (1965 a). A steady stimulus may thus produce a heightening of the excitatory state although it is insufficient to maintain it at a suprathreshold level. This will be true during physiological standard conditions such as in the present experiments in which the routine was to avoid any deformation prior to stimula-

tion. It is interesting, however, that with a high degree of stretch, which is probably not physiological, the adaptation may be counteracted to such an extent that a stationary discharge is obtained from the previously rapidly adapting touch receptors (LOEWENSTEIN 1956 a).

The various signs of enhanced excitability during steady deformation show that mechanical recovery of the receptive structures, if it occurs within the deformed skin, is at least incomplete and may not be the only basis for the rapid adaptation of these receptors (cf. DUN and FINLEY 1938, LOEWENSTEIN 1956 a, GRAY and SATO 1953 and EYZAGUIRRE and KUFFLER 1955). This may also be due to rapidly declining responsiveness of the mechano-sensitive structure while it is still in a deformed state, to high accommodation in the axon ending supplying the receptor (cf. GRAY and MATTHEWS 1951), or to chemical inhibition (HOAGLAND 1936). The present experiments have not given any direct information about the mechanism behind the adaptation. The behaviour of the receptors during the moving phase and the plateau phase of the stimulation suggest, however, that a dual mechanism, or at least two different properties, may be involved both in excitation and adaptation. One mechanism or property may be responsible for the initial discharge and another for the discharge or subliminal change of excitability during the staedy phase of the stimulation. A comparison may be drawn with the dynamic and static components of the discharge from muscle spindles (KATZ 1950). In these receptors the mechanism or property responsible for the static component is naturally prevalent, while in touch receptors, whose primary task is to signal rapid superficial displacements rather than tension, it seems to manifest itself mainly under special circumstances such as high stretch (LOEWENSTEIN 1956 a) or changes in the chemical environment (TALAAT 1933).

The critical slope was between 0.8 and 40 mm/sec for one third of the receptors and for the rest it was of or below 0.8 mm/sec. These values are of the same order of magnitude as those obtained by GRAY and MALCOLM (1951) although different techniques have been used. Gray and Malcolm, working on nerve-skin preparations, applied their stimuli to the inside of the skin so as to stimulate the termination of a single axon, while in the present investigation the receptors were studied in situ and the stimuli were applied to the skin surface over individual receptors. In their experiments Gray and Malcolm also measured the threshold at various stimulus gradients and the curve shown by them (GRAY and MALCOLM 1951, Fig. 5) follows the same course as that in our Fig. 4.

It has been shown that the threshold does not decrease progressively with increasing gradient of the stimulus except for gradients near the critical slope. This fact, which may be looked upon as a limitation of the receptor to respond to rapid stimuli, may be due to inertia of the tissue through which the mechanical forces have to be transmitted or of the receptor itself. It is known, however, that the minimum latency of the initiation of an impulse in these receptors is very short; according to GRAY and MALCOLM (1951) it is of the order of

1 msec. This and other circumstances, such as the rapid recovery of the skin (LINDBLOM 1958) and the effectiveness of vibratory stimulation (CATTELL and HOAGLAND 1931), indicate that the inertia is slight and that the receptor is capable of reacting rapidly. The relative insensitivity to variations in the rate of deformation may therefore be an inherent property of the receptor which has no simple physical explanation. It may be the axon ending in the receptor that is responsible for the insensitivity. TASAKI (1950) has shown that the same type of threshold-gradient curve is obtained on electrical stimulation of the toad's nerve fibres.

On *suprathreshold* stimulation an increase of the gradient was accompanied by a shortening of the latency and an increase of the frequency of the discharge. Concomitantly the duration of the discharge was reduced, mainly because an increase of the gradient alone means that the effective moving phase of the stimulus becomes shorter. This sequence of changes will be generally valid if it is assumed that the amplitude of stimuli occurring in natural, as well as experimental, situations has to be limited. On stimulation with a steep gradient the adaptation will have relatively less time to exert its action and this would seem to result in a relatively greater total number of impulses. This may not be true, however, because the refractory state summates during repetitive discharge and becomes a more powerful limiting factor the higher the firing frequency tends to be (cf. LINDBLOM 1958). The ultimate frequency and duration of the impulse discharge, therefore, will be dependent upon the resultant action of the characteristics of the stimulus and the various excitability properties of the receptor and axon ending.

The temporal fluctuations of the critical slope, as well as the similar fluctuations of the threshold (LINDBLOM 1958), will diminish the exactness with which outer stimuli will be reproduced, since the afferent discharge elicited by identical stimuli will vary slightly in an unpredictable way. The temporal fluctuations may be random variations in excitability, but they may also represent an integrative mechanism, which would be a centrifugal regulation of the sensory input at the receptor level (cf. LOEWENSTEIN 1956 b). It may be mentioned here that there are also spatial variations in excitability within the sensory unit, since the various receptors supplied by the same afferent fibre usually have different thresholds (LINDBLOM 1958). In comparison with the temporal and spatial variations in excitability, the limited capacity of the receptor to follow changes in the characteristics of the stimuli, as *e.g.* the relative insensitiveness to variations in the gradient, may be subordinate.

All receptors have been classified as low-threshold rapidly-adapting receptors, but it should be pointed out that there are wide variations in the rate of adaptation and the threshold from one receptor to another which are far outside the range of the temporal fluctuations. All intermediate forms seem to exist between the very rapidly and the less rapidly adapting receptors, as well as between those with a relatively high and a relatively low threshold. It is possible that the

most rapidly adapting receptors with a critical slope of about 100 rheobases per second or more (cf. table 1) constitute a subgroup which should be separated from the rest of the receptors. This would be in line with the findings of DUN and FINELEY (1938) and FESSARD and SEGERS (1942) and also with the impression gotten by MARUHASHI, MIZUGUCHI and TASAKI (1952) that there are two kinds of tactile end-organs in the toad's skin, one "phasic" and one "semitonic". A close comparison with the classification proposed by other authors is not possible since different techniques and criteria have been used. Several selective factors are also involved. Thus, in the present material one such factor is the dissection, which may have led to an over-representation of fibres resistant to mechanical injury, and another that only units innervating the lower leg have been included. Further, the receptors having a relatively high or a very low threshold, or a critical slope below 0.8 mm/sec, could only be subjected to an incomplete analysis. As far as can be judged, these receptors have the same general properties as those which could be studied through the whole range of slopes and amplitudes of displacement. As yet, however, it cannot be decided, at least as far as the toad is concerned, whether there are any subgroups of functional significance within the population of tactile units with coarse afferent fibres.

The critical slope for discharge and the duration of the discharge on suprathreshold stimulation may be taken as two different measures of adaptation. It would be interesting to compare these two data to see how close the correlation is. Although the technique we have used makes such a comparison possible the material so far analyzed is too small to allow any conclusion in this respect. Nor can it be decided whether the critical slope is correlated to the rheobase value.

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A Presynaptic Effect of d-Tubocurarine in the Neuromuscular Junction

By

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Abstract

LILLEHEIL G. and K. NAESS. *A presynaptic effect of d-tubocurarine in the neuromuscular junction.* Acta physiol. scand. 1961. *52.* 120—136. — End-plate potentials (e. p. p.) of isolated phrenic nerve diaphragm preparation have been recorded at different stages during development to complete curarization and during recovery of the preparations. The first effect obtained during increasing curarization is characterized by the constancy of the e. p. p. during tetanic stimulation, *i.e.* by lack of Wedensky inhibition. This phenomenon, with a rapid decline of e. p. p., develops under the influence of tubocurarine and must probably be caused by this pharmacological influence. It is concluded that the mechanism behind the Wedensky inhibition must be of presynaptic origin, and probably be due to a reinforcement of the naturally occurring tendency to block of propagation of tetanic impulses in the last arborization of the motor nerve.

The following topics are more comprehensively treated in the discussion: 1. The possible presynaptic effect of tubocurarine in relation to the classical theory of a pure postsynaptic one demonstrated by DALE, FELDBERG and VOGT (1936) when single stimulation were used. 2. The possible course of release of acetylcholine during tetanic stimulation. 3. Possible desensitization of receptor substance to ACh during transmission of repetitive impulses. 4. Effect of other quaternary ammonium compounds on nerve fibres, especially on unmyelinated nerve endings.

Since the work of DALE, FELDBERG and VOGT (1936) the effect of (curarine) d-tubocurarine has been believed to be due to a pure postsynaptic mechanism — the competition between acetylcholine (ACh) and the drug for the receptor

substance. These authors found no change of output of ACh under the influence of curarine. Tetanic stimulation was, however, not used in this investigation, and the work gave, therefore, no clue to the understanding of the mechanism behind the Wedensky inhibition — a phenomenon so characteristic for the effect obtained by curarization.

Since 1936 a series of papers on the effect of tubocurarine and investigations in which this drug has been used as a tool have been published. Tetanic stimulation has, as a rule, been used in these publications and the discussions and conclusions have generally been based on the assumption that curare produces no change of output of ACh even during tetanic stimulation. This assumption has, however, never been proved. Some papers mentioned in the discussion have, on the other hand, given some indication of an additional presynaptic effect of tubocurarine, but these papers have never received much attention.

The purpose of this investigation has been to submit this possible presynaptic effect of tubocurarine to a closer investigation. The main result of the investigation is that the mechanism behind the Wedensky inhibition produced by tubocurarine must be of predominantly — if not exclusively — presynaptic origin. Our findings are comprehensively discussed in relation to relevant information concerning the mechanism for synaptic transmission, and particular attention is paid to the question of variation of release of the transmitter under physiological conditions.

A preliminary report of our results has been published recently (Lilleheil and Naess 1960).

Methods

Isolated rat phrenic nerve diaphragm preparations have been used. Action potentials have been recorded with two electrodes, one in the central part of the motor end-plate region and the other a few mm away on the same fibres. The first one records the end-plate potential (e. p. p.) under normal conditions with a diphasic action potential superimposed, and after appropriate curarization a pure monopolar end-plate potential, while the other one records a spike potential from the muscle fibres outside the neuromuscular junction. The potentials are recorded with the preparation submerged in a bath of oxygenated (+ 5 % CO_2) Tyrode's solution. Rectangular supramaximal stimuli of 0.3 msec were applied to the phrenic nerve, either as single impulses with a frequency of 1/sec or as tetanic stimulation with a frequency of 50/sec, which is believed to be approximately the maximal naturally occurring frequency in this preparation. The traces on the cathode ray screen have often been blanked for time intervals of 1/3 sec under tetanic stimulation to have the potentials clearly separated for publication. Continuous registration on film has also been used.

Location of the end-plate zone is a relatively simple procedure when the following method and criteria are used: 1. The macroscopically visible end-plate zone observed in the partly transparent preparation transilluminated from below. 2. The shortest latency between the stimulus artefact and the start of the potential, and the characteristic change of shape of the potentials when passing the electrode across the end-plate zone and the adjacent region. 3. The typical monopolar potential obtained after "complete

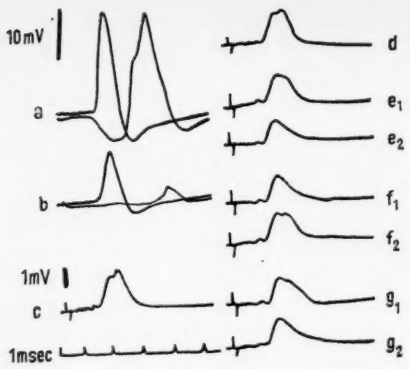

Fig. 1. Action potentials from rat diaphragm preparation after indirect, supramaximal stimulation of phrenic nerve. a) Single stimulation before curarization. First potential from end-plate zone, second potential from the muscle fibre a few mm away from this zone. b) 8 min after addition of 3 μg/ml d-tubocurarine chloride to the bath. c) Potential from end-plate zone after single stimulation 14 min after curarization. Note increase in amplification for this and the following recordings. Muscle fiber action potential has now disappeared, and the beam for recording of this potential has been switched off. d—g) Some of the action potentials recorded with the same electrode as in c during a tetanic stimulation (50/sec) immediately following c. d = First potential, e_1 and e_2 = after 1 sec. f_1 and f_2 = after 8 sec. g_1 and g_2 = after 16 sec.

curarization". 4. As an additional control, the highest potential after disappearance of the spike potential.

d-Tubocurarine chloride ("Nyco") has been added to the bath. The term "complete curarization" is in this paper used for a stage when the spike potential has disappeared, leaving the local non-propagated potential in the end-plate zone.

Results

The results are presented in 3 figures, the two first ones demonstrating in their purest form the two typical effects obtained at different stages of curarization. The third demonstrates more continuously the development of the effect after addition of tubocurarine and during recovery of the preparation.

The first recording in Fig. 1 demonstrates the normal potentials before curarization, the second one the potentials just before "complete curarization".

The last recording in the first column is only the potential in the motor end-plate zone when the superimposed spike potential is on the verge of disappearing. The potential recorded is now predominantly a pure monopolar e. p. p. The second column represents recordings of e. p. p. from the same place during tetanic stimulation with 50 st./sec immediately after the single stimulation. It is obvious that the e. p. p. is maintained on an approximately steady level even during a long tetanic stimulation. A typical effect of this kind can only be obtained in relatively few preparations during the introductory phase of the effect of tubocurarine and only when an appropriate concentration of tubocurarine has been used for the preparation under investigation. A steady level of the e. p. p. can under such circumstances be obtained from approximately 5—15 min after addition. With higher concentrations, or after a longer period of action of tubocurarine, the potentials are reduced more or less during the

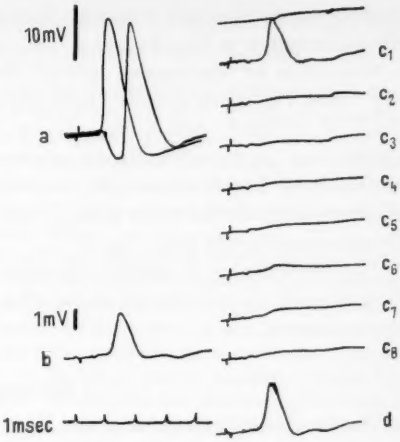

Fig. 2. Similar potentials as in Fig. 1. a) As in Fig. 1 a. Between a and b addition of 0.2 μg/ml d-tubocurarine chloride. Disappearance of muscle fiber action potential after 14.5 min and complete disappearance of end-plate potential after 18.5 min. Washing out of tubocurarine 33.5 min after curarization. b, c, and d recorded 15 min later during recovery of the preparation from the complete block, just before the first muscle fibre potentials reappeared. b) Potential from the end-plate zone after single stimulation. c_1—c_8) Some of the end-plate potentials recorded with the same electrode as in b during tetanic stimulation (50/sec) immediately following b. c_1 first potentials and the other with intervals of 1/3 sec. d) As in b, 3 sec after determination of tetanic stimulation. Note increase in amplification between a and b.

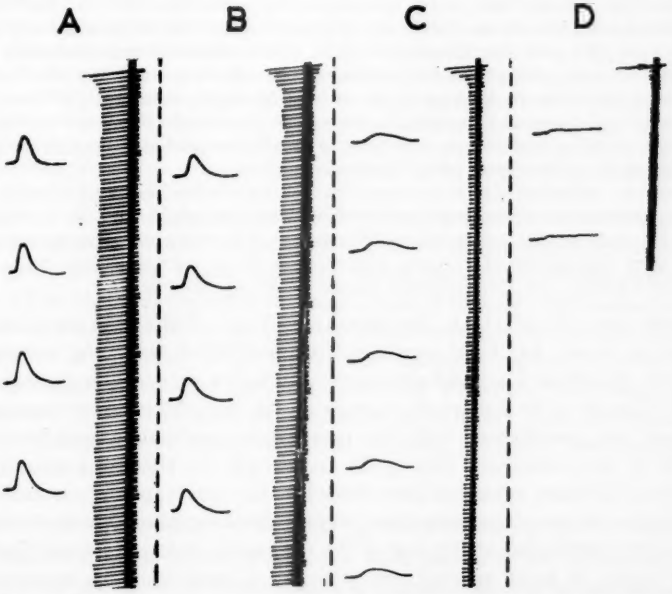

Fig. 3. Changes of e. p. p. during tetanic stimulation with 50/sec under increasing curarization (0.2 μg/ml d-tubocurarine) and during recovery of preparation. Continuous recording of e. p. p. downwards along the film combined with simultaneous registration of some of the same e. p. p. across the film with intervals of approximately 0.5 sec. A: On the verge to "complete curarization", B and C: During increasing curarization, D: During recovery after complete disappearance of all e. p. p. and washing out of tubocurarine. Relative amplificaton: A and B: 1, C: 1.3 and D: 2.3.

tetanic stimulation and represent then transitory stages to the other typical effect illustrated in Fig. 2.

Very often an overlapping between the two typical effects demonstrated in Fig. 1 and Fig. 2 has already developed when the stage of "complete curarization" is obtained, and quite constant potentials can, under these circumstances, not be recorded during tetanic stimulation. The typical picture demonstrated in Fig. 2 is also not a quite constant finding, but a rapid decline of the e. p. p. always takes place in this phase, even if the potentials do not disappear so rapidly as in Fig. 2.

The development in another characteristic experiment can be followed more closely in Fig. 3, which requires no other comment than the information given in the legend.

Discussion

The Wedensky inhibition of tetanic contractions has always been considered a typical effect of tubocurarine and substances with similar action.

Wedensky demonstrated in his comprehensive work from 1903 the inhibition of conduction and transmission of impulses produced by repetitive stimulation with higher frequencies. He, and also Hofmann (1903), proved that the neuromuscular transmission was more sensitive to this inhibition than the nerve and the muscle fibres. This naturally occurring limiting factor could be strongly enhanced by different types of physical and chemical influences. In the motor end-plate the inhibition was increased by ether, nicotine and curare. All types of inhibition produced by high frequency stimulation have since been called Wedensky inhibition.

Wedensky inhibition of tetanic contraction is usually defined as the phenomenon that these contractions are transformed to very short-lived ones, which are only recorded as a twich by usual mechanical recording. This is a type of contraction seen during a relatively deep curarization of a certain duration (see Naess 1952 and 1953) but slighter forms of inhibition are also sometimes named Wedensky inhibition.

Since the work of Dale, Feldberg and Vogt (1936), the mechanism of action of curare has been considered an established fact. The competition between the released acetylcholine and the drug for the receptor substance has been believed to be the whole explanation of the inhibition of transmission, without any interference with the presynaptic production, mobilization or release of the transmitter. During the last decade the effect of a series of substances of different types has been traced to the presynaptic fibres and/or the mechanism of acetylcholine-release. Papers demonstrating this mechanism of action are mentioned at the end of the discussion. Before the final discussion some topics of more general and principal interest must be surveyed and discussed.

1. *Discussion of the work and conclusion of* Dale, Feldberg *and* Vogt

In the description of their method these authors give valuable information concerning certain procedures which may limit the possibilities of too far-

reaching conclusions concerning the mechanism of action of tubocurarine. This technical information has never been paid sufficient attention.

It is stated that acetylcholine could only be obtained in the perfusion fluid during stimulation when saline solution was used. When animals were pretreated with eserine and atropin and perfused with blood, no acetylcholine could be found. A certain possibility of working with anoxic muscles must exist when saline solution is used, especially when the muscle is stimulated for longer periods. The neuromuscular transmission and the release of acetylcholine is probably most easily affected by lack of oxygen (WEDENSKY 1903, NAESS and STORM MATHISEN 1955).

DALE *et al.* mentioned that the artificial conditions of the saline perfusion in some way facilitate the escape of acetylcholine from the site of its origin into the blood vessel. FLEISCH, SIBUL and KAELIN (1936) found that acetylcholine appeared even when the preparation was perfused with blood, if only the blood was not saturated with oxygen. It must be generally accepted that a preparation on the verge of anoxia is not the best one for a more complete elucidation of all details in the mechanism of drug action, even if the main features of the action may be correctly demonstrated. (For a further discussion of the limited significance of ACh appearance in junctional perfusate, see NACHMANSOHN 1959 and section 4 in this discussion.)

DALE *et al.* also mentioned that the use of saline solution caused a limitation of the experimental period in which success was possible. If the period was too long the muscle became *quickly* insensitive to motor nerve impulses. There is, therefore, reason to believe that the experiments must have been done in a limited period and that the effect of curarine was tested as soon as the transmission had been blocked after the injection of the drug. Previous work (NAESS 1952) and the present results demonstrated that the complete effect of tubocurarine, including what we believe is a presynaptic effect, takes considerably longer time to establish itself than the rapidly occurring postsynaptic one. Experiments of short duration may, therefore, not reveal all the effects of tubocurarine.

A factor of central significance will be mentioned at the end of this section: Tetanization was not used in the work of DALE *et al.* The frequency of stimulation never exceeded 15/sec and single contractions down to 5/sec were often used. "This method, causing a rhythmic series of twitches, was chosen in preference to tetanization, so as to avoid impediment to the perfusion during activity of the muscle." These frequencies cannot — especially during introductory stages of curarization — produce any Wedensky inhibition, and the mechanism behind this phenomenon has, therefore, not been investigated by DALE, FELDBERG and VOGT. This has, however, obviously been assumed by several other workers.

2. *Previous literature dealing with the release of transmitters during repetitive stimulation*

A chapter dealing especially with the ACh release in the motor end-plate during transmission of repetitive stimuli is necessary for the further discussion. Possible changes in the ACh-production in a short interval at the beginning of such transmission can not be directly measured (see p. 130). This is, however, the period of importance in connection with the Wedensky inhibition. It is, therefore, of interest to look at the characteristic features of blocks produced by other procedures than curarization. The lack of Wedensky inhibition of the

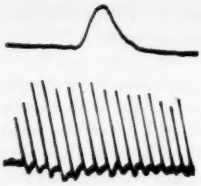

Fig. 4. E. p. p. produced by single stimulation and a short tetanic stimulation with 50/sec after block of transmission by addition of 6.10^{-3} M $MgSO_4$ to the bath. (Single and repetitive e. p. p. recorded with different time base.)

reduced tetanic contractions of tibialis ant. muscle of cat when depolarizing substances are used, indicates a relatively constant release of ACh per impulse. This assumption cannot be tested in rat diaphragm preparation because the effect of depolarizing substances is so rapidly transformed to a curare-like effect with a pronounced Wedensky inhibition. Procedures which reduce the release of ACh, excess of magnesium, lack of calcium and use of botulinum toxin, produce inhibition which is characterized by the opposite phenomenon of a Wedensky inhibition, *viz.* facilitation through a block. Tetanic contractions increase their amplitudes with increasing frequencies of stimulation, and no drop in amplitude occurs during the stimulation.

Recording of e. p. p. under such circumstances reveals the facilitation as demonstrated by use of magnesium in this paper (Fig. 4). This implies a progressive increase of the ACh release per impulse during the first period of stimulation. Block produced by excess of sodium gives no Wedensky inhibition, and a slight increase of e. p. p. or stable e. p. p. are obtained by this procedure. Low sodium block also gives constant amplitude of e. p. p. during the first period of stimulation (See section 3).

Inhibition of neuromuscular transmission undoubtedly plays a role in the reduction of more protracted tetanic contractions produced under physiological conditions. Such contractions reduce or completely stop the circulation through the muscle simultaneously with an increased requirement of oxygen. The asphyxia depresses the transmission and is the limiting factor for the strength and duration of the maximal tetanic contraction. (For a closer discussion see Wedensky (1903), Naess and Storm Mathisen (1955).)

The question of interest in connection with our present problem and work is, however, whether a sufficient and rapid reduction of ACh output per impulse also takes place during tetanic contractions of short duration, *e.g.* up to 5 sec. This is absolutely necessary if the Wedensky inhibition seen under curarization shall be explained by the generally accepted theory of Rosenblueth and Morison put forward in 1937. Their suggestion of a rapidly declining ACh-production during transmission of tetanic impulses, at that time, based on predominantly hypothetical considerations. The following authors have, up to the present time, been repeatedly quoted in support of the above mentioned theory: Brown (1937), Eccles, Katz and Kuffler (1941), Liley and North (1953).

BROWN states in his paper: "BROWN and DALE (1937) have evidence that in a succession of nerve impulses each successive impulse liberates progressively less of transmitter if they are sufficiently close together." This refers to unpublished results, which do not allow closer evaluation. We do not know the frequencies used to obtain a reduction, or whether the investigation — like the following ones — has been made under curarization, which with our present knowledge must be considered a serious objection against this statement.

ECCLES *et al.* and LILEY and NORTH have used curarized preparations. The fact that the e. p. p. decreases during tetanic stimulation in curarized preparations cannot, however, on the following grounds give any certain information of the processes under normal conditions. All authors who have taken this reduction as a proof of a progressive decline of the ACh-production have accepted the statement of DALE *et al.* of a pure postsynaptic effect of curarine as an absolute fact. As stressed earlier, DALE *et al.* have, however, never made any investigation of the ACh-release during tetanic contractions (see section 1).

HUTTER (1953), who made well-founded investigation of the facilitation and inhibition of the neuromuscular junction, was of the opinion that his results had proved the hypothesis of ROSENBLUETH and MORISON (1937). He demonstrated that the ACh-sensitivity of the motor end-plate did not change during a Wedensky inhibition. He consequently concluded that the Wedensky inhibition must be due to a pure presynaptic mechanism of action. We certainly agree in this conclusion, but cannot accept the result as a proof of a naturally occurring decrease of ACh production under physiological conditions as suggested by ROSENBLUETH and MORISON. HUTTER's conclusion is, thus, also based on the statement that tubocurarine has no presynaptic effect, an assumption which is never proved and — we believe — disproved by our present work.

The experimental results of HUTTER are, however, of great value and form a cornerstone in our main discussion (section 3).

KRNJEVIĆ and MILEDI (1958) have made a comprehensive study of the neuromuscular block produced by long sustained tetanic stimulation with different frequencies. Their result has also been quoted as support of a theory of pre-junctional deficiency in the release of the transmitter substance. Their experiments gave, however, no proof of an exhaustion of ACh as the mechanism behind the inhibition of transmission obtained in their experiments.

Microelectrodes were introduced intracellularly during tetanic contractions which, as a rule, had gone on for a long period before registration of e. p. p. was started. This investigation can, therefore, give no information of a possible naturally occurring deficiency in the release of the transmitter in the first period of transmission of tetanic impulses. The very reliable results of these authors demonstrate that two types of block are at work during long sustained tetanic contractions, a presynaptic failure of propagation and another of postsynaptic origin. These types of block develop after a relatively long period of stimulation, and are irrelevant in connection with our present problem. The relatively low safety factor for conduction in the unmyelinated presynaptic fibres is, however, of special interest in connection with our final discussion of the mechanism behind the presynaptic effect of tubocurarine (section 5 b).

The conclusion of this survey of previous relevant literature must therefore be: There is no direct or indirect proof of a rapid and progressive reduction of the ACh release per impulse during transmission of tetanic impulses, *i.e.* no proof of a naturally existing basis for the Wedensky inhibition, which is especially characteristic for curarization.

The increase of e. p. p. in the beginning of a tetanus in frog during curarization (Eccles *et al.* 1941) is also worth mentioning. There must probably be an increase in the release of ACh per impulse in this preparation and no rapid depletion as assumed for the mammalian preparations. We believe that the difference observed by curarization of the two types of preparation is due to a more slow presynaptic effect of the drug in frog. This would make the pure postsynaptic effect of curare more readily observable in this species of animal.

It may be of interest to look for other processes of transmission, which can be observed in more detail without interference by any drug. R. Eccles (1955) has recorded synaptic potentials by microelectrodes in ganglia of rabbits. The stability of these potentials, even with relatively higher frequencies (up to 40—50/sec) was remarkable in this investigation too. No significant decline in the amplitude took place in the beginning of the transmission of repetitive stimuli, and there seems therefore, not to be any decrease of ACh release in this transmission when limited periods of stimulation were used.

The stability of the postsynaptic excitatory potentials recorded from the motoneurons of the spinal cord during high-frequency stimulation of afferent fibres has been demonstrated by Curtis and Eccles (1960). These authors assume a general mechanism for different types of transmission by which each impulse causes a depletion of transmitter simultaneously with a mobilization of new transmitter from the store. With somewhat higher frequencies of stimulation the last process may compensate or even overcompensate the first one. The following is stated with regard to the motoneurons: "In this way high frequency of synaptic activation calls forth a mobilization of transmitter that ensures a greater synaptic effectiveness for rates of synaptic activation up to 250/sec". An increase may even be seen in the excitatory synaptic potentials recorded intracellularly by microelectrodes with frequencies from 80—125/sec. The release of the transmitter in these synapses must obviously have a high safety factor with respect to transmission of repetitive stimuli.

It is not easy to understand why motor end-plate should be an exception, with a *rapidly* declining release of transmitter under physiological conditions, and we are — as already stated — of the opinion that a series of direct evidence seems to disprove this assumption. (See also addendum.)

3. *Desensitization of the motor end-plate to ACh during tetanus*

Another hypothesis put forward by Thesleff (1959) which could partly explain the Wedensky inhibition must also be mentioned and briefly discussed. Thesleff used hypertonic NaCl solution to block the neuromuscular transmission and found a relatively slight reduction of e. p. p. combined with a sometimes strong and rapid reduction of the sensitivity of the motor end-plate to electrophoretically applied ACh. The consistent conclusion was the assumption of a rapid desensitization of the motor end-plate to ACh during tetanus. This could, according to Thesleff, partly explain the mechanism behind the

phenomenon of Wedensky inhibition. Some of his findings and the hypothesis have been tested and discussed by the present authors in a special paper (NAESS and LILLEHEIL 1961). The main result of this paper was the constancy of amplitude of e. p. p. during a tetanus recorded immediately after the preparation was blocked by excess of sodium. Mechanical recordings of tetanic contractions during increased NaCl concentrations, did not show any sign of Wedensky inhibition either. THESLEFF's observation made with the addition of NaCl can, therefore, probably not explain the mechanism behind the phenomenon of Wedensky inhibition. The reader is referred to the paper of LILLEHEIL and NAESS (1961) for a more comprehensive discussion. Three other experimental results which are not in agreement with the hypothesis put forward by THESLEFF, however, will be especially mentioned here:

HUTTER (1953) tested the ACh-sensitivity of the motor end-plate to ACh before and during a completely Wedensky-inhibited tetanic contraction in a curarized preparation. He found no change in the sensitivity, and concluded that such a mechanism could not explain the inhibition.

Quite recently OTSUKA and ENDO (1960) using microelectrodes and electrophoretically applied ACh in curarized frog preparations confirmed this result and could not support the finding of THESLEFF indicating a rapid desensitization of end-plate to ACh. LILEY (1956) observed the miniature potentials during and immediately after transmission of tetanic impulses and found no change in the amplitude of these potentials. Once again the conclusion must be drawn that probably no change in the ACh-sensitivity occurs during the first part of tetanic stimulation under physiological conditions, even if this is the case during more prolonged tetanic stimulation (KRNJEVIĆ and MILEDI 1958).

4. *Main discussion of present results*

The stability of the e. p. p. in the first seconds of tetanus in the first phase of curarization must, according to the present results, be considered a fact.

It is not possible to obtain quite constant amplitude in the introductory phase in all preparations, but the decline is not more than about 20 per cent in 10—15 sec when the correct concentration of tubocurarine and the correct time for registration are chosen. There are in this repect certain individual variations dependent on the age of the animal and thickness of the preparation. The last factor is of significance for obtaining a rapid and even effect in all fibres from which the extracellular electrode records.

The Wedensky inhibition of the e. p. p., *i.e.* the rapid decline in the potentials, develops progressively under the influence of tubocurarine, but is to a certain extent covered by the simultaneous reduction of all potentials. The rapid decline of the e. p. p. is, therefore, most readily observed during the recovery of the preparation after tubocurarine has been washed out. The unchanged ACh-sensitivity of the motor end-plate during tetanic stimulation (see section 2 and 3) entails that the relative inhibition of e. p. p. produced during tetanus must be considered to be of presynaptic origin and the result of the experi-

mental procedure *i.e.* curarization. *Tubocurarine must have produced this effect itself in its second phase of action.* This conclusion is the same as stated previously from results obtained by mechanical recordings of muscular contractions in rabbits (NAESS 1952, 1953).

The different time course of production of the pre- and postsynaptic effects, which make the separation of the two mechanisms possible, must probably be due to different penetrability of tubocurarine into the two structural membranes concerned — the postsynaptic membrane being the most readily affectiable.

It has previously been demonstrated that the postsynaptic effect reaches its maximum 3—4 min after intravenous injection in rabbits and the possible presynaptic effect is fully developed after 15 min when continuous injection is used (NAESS 1952). It is not so easy to give exact information concerning the time factor when rat preparations are used, due to the more pronounced overlapping of the effects when the drug must penetrate into the preparation and thus is administered more slowly and at various speeds to the different end-plates.

The finding that the Wedensky inhibition is most distinctly observed during the recovery of the preparation (see Fig. 2 and 3) is according to our opinion probably due to the more rapid removal of tubocurarine from the postsynaptic bindings. This will reduce the competitive postsynaptic inhibition more rapidly than the presynaptic one, which is responsible for the Wedensky inhibition.

A direct measurement of the ACh-output before and during curarization may, perhaps, seem necessary to substantiate our postulate of a presynaptic effect of tubocurarine. We will, therefore, explain the reason why we do not rely on this method when tetanic stimulation is used:

The difficulties encountered by DALE *et al.* with perfusion of muscles *in vivo* (see section 1) are almost an adequate explanation. Use of isolated preparation and examination of the output of ACh in the bath during long sustained tetanic stimulation may seem to be a more reliable method. Long sustained tetanic stimulation necessary to produce sufficient amounts of ACh in the bath is, however, a questionable method, because it causes the neuromuscular junctions to work under unphysiological conditions, which to a certain degree are similar to those produced when the muscle is perfused and stimulated *in situ.* (For further discussion of significance of variation in measurable ACh see NACHMANSOHN (1959) pp. 206—208.)

Addition of inhibitors of cholinesterase to the bath is necessary. The ACh produced is not split, and lack of choline with significant influence on the restoration of the presynaptic store of ACh may ensue. Excess of ACh around the presynaptic fibres can certainly interfere with the possibilities of other quaternary ammonium ions, *e.g.* tubocurarine, to interfere with the activity of the presynaptic fibres (see different presynaptic effect of quaternary ammonium ions p. 132 and possible presynaptic effect of ACh mentioned in the last section of the discussion (section 5 c). Inhibitors of cholinesterase and structually related substances seem by themselves to have certain presynaptic effects (see RIKER *et al.* 1957, RIKER *et al.* 1959, NACHMANSOHN 1959, p. 198), which may interfere with the transmission of tetanic impulses and produce unphysiological basic conditions for this type of experiment.

It is also of interest to mention the recently demonstrated effect of adrenergic blocking agent on the "overflow" of noradrenaline into the perfusion fluid (BROWN

1960) and the hypothesis of a presynaptic effect of this type of drugs put forward by PATON (1960) in the discussion of this result.

A series of arguments thus limit the reliability of results obtained in this way, and we found no reason to perform experiments of this type to support or disprove our theory. Direct measurement of the e. p. p., combined with relevant information from other reports is, in our opinion, a far better and more trustworthy procedure, especially with regard to the first short interval of transmission of impulses — the period of interest for the actual problem — the mechanism behind the Wedensky inhibition in curarized muscles.

A series of previous results have given certain indications of a presynaptic effect of tubocurarine. In a few instances a theory including a presynaptic effect of tubocurarine has been proposed on the results obtained by ABDON and BJARKE (1945), NAESS (1952, 1953), and the last time quite recently by RIKER *et al.* (1959).

Relevant papers are briefly quoted here:

BUCHTHAL and LINDHARD (1942) investigated the function of isolated motor end-plates of the lizard. Though they demonstrated the specific sensitivity of the motor end-plate to ACh, and also showed the reduction in this sensitivity produced by curarine, they found by means of electrical stimulation that the first boundary of the motor end-plate, the neurogenic component, was blocked at a time when the excitability of the myogenic component, the second boundary, was not changed. After acting for some time, however, curarine also blocked this boundary.

ENGBÆK (1948) has repeated this experiment and confirmed the results. She gives information on the concentration necessary to block the two components of the motor end-plate. ENGBÆK mentions that the results suggest a blocking of terminals of the motor nerve, but dismisses this possibility owing to the then accepted fact that curare does not influence the activity of peripheral nerves even at high concentrations. (See however, DETTBARN (1960), GRUNDFEST and NACHMANSOHN (1952)).

ABDON and BJARKE (1945), by a biochemical method, found no production of free acetylcholine after indirect tetanic stimulation of a frog muscle, while direct stimulation still produced normal amounts. The method of these authors has, however, been criticized and it has been difficult to use in the hands of other scientists. (V. P. WHITTAKER and others, personal communication.)

LAPORTE and LORENTE DE NÓ (1950) have demonstrated that an increase in the L-fraction of the membrane of presynaptic fibres of the sympathetic ganglion of turtle takes place during curarization. From earlier observations of LORENTE DE NÓ it is known that an increase in the L/Q ratio of membrane potentials will produce a block which will be reinforced by long trains of impulses. (See also section 5 b.)

NAESS (1952, 1953) concluded on the basis of changes in the form of myograms obtained under development of curarization in rabbits that tubocurarine presumably had a presynaptic effect in addition to the postsynaptic one.

RIKER *et al.* (1957) demonstrated that certain phenolic quaternary ammonium ions facilitate neuromuscular transmission primarily by an action on the motor nerve terminals. The presynaptic effect was especially specific for 3-hydroxyphenyl-triethyl-ammonium (3-OH PTEA). It was demonstrated in the same paper that small doses of tubocurarine and also procain, well below those that interfere with single-volley transmission, obliterate the repetitive antidromic activity in the nerve produced by 3-OH PTEA. Hence, it was concluded that the blocking effect of curare in the motor end-plate occurs

primarily at the motor nerve terminals. It is, for the further discussion, of interest to mention that the repetitive firing produced by 3-OH PTEA and blocked by tubocurarine usually was of high frequency (about 100/sec or above).

Our investigation gives no information which can support the hypothesis of RIKER *et al.* of a *primarily* presynaptic effect of tubocurarine. *The importance of the well-established postsynaptic competitive effect of tubocurarine* (DALE *et al.*) *is of course fully accepted by us. The presynaptic effect, demonstrated in the present paper, comes in addition to the postsynaptic one.* The former can be observed especially — or perhaps only — during transmission of tetanic impulses. The higher the frequencies of stimulation, the stronger is this effect. The investigation of DALE *et al.* has forever demonstrated that the postsynaptic effect is the dominant one when single stimuli are used. Nothing in our results is at variance with this well founded and generally accepted investigation. The effect of small doses of tubocurarine on the repetitive discharge of presynaptic fibres produced by special substances — as demonstrated by RIKER *et al.* (1959) has also primarily revealed an inhibitory effect on the ability of production and conduction of repetitive impulses of higher frequencies. The result is, thus, also in agreement with our results and conclusion. We are of the opinion that the indirect evidence of a presynaptic effect, demonstrated by RIKER *et al.* (1959), can not prove anything certain regarding a possible presynaptic effect of tubocurarine on transmission of single impulses under normal conditions. It seems at present, most reasonable to trust the classical work of DALE *et al.* in this respect.

5. *Possible mechanism of presynaptic action of tubocurarine*

Very little is known about the finer mechanism of conduction or electronic spread of impulses of the smallest presynaptic fibres and of the release of ACh. It is, therefore, impossible to give a more detailed theory for the mechanism behind the presynaptic effect of tubocurarine. Some factors of interest in this connection ought, however, to be mentioned here:

a. *Penetrability of quaternary ammonium ions into the nerve fibres, especially presynaptic ones*

It was, until quite recently, the general opinion that quaternary ammonium ions due to their low lipid solubility and completely charged state, could not penetrate into nerve fibres, including the smallest ones. Different results obtained by various workers during the last three years have, however, demonstrated that the finest terminals of efferent as well as afferent nerves differ from the main part of the myelinated nerve fibre in this respect: STOVNER (1958 a) and KOTETSU (1958) demonstrated independently that tetraethyl ammonium affected the presynaptic fibres and increased the output of ACh per impulse, thereby producing an antiblocking effect on different types of inhibition of the neuromuscular transmission (see also STOVNER (1958 b)). RIKER *et al.* (1957, 1959) demonstrated an ability of special quaternary ammonium ions to produce repetitive discharge in the presynaptic fibres, and RIKER and SZVENIAWSKI (1959) achieved a corresponding effect on the pre-

synaptic fibres in the sympathetic ganglion. The latter authors were also of the opinion that they could demonstrate a depressive effect of certain ganglionic blocking substances, among others hexamethonium, on these presynaptic fibres.

It has also been shown that juxtaarterial injection of ACh can stimulate the terminals of sensory fibres and this effect can be readily blocked by hexamethonium (DOUGLAS and RITCHIE 1960). The new adrenergic blocking compound, bretylium, which is also a quaternary ammonium ion, acts on the postsynaptic sympathetic fibres, and the strongest effect seems to occur on the nerve endings (GREEN 1960). Even cholinesterase inhibitors of the quaternary ammonium group seem to have certain presynaptic effects (for references see RIKER *et al.* 1957, NACHMANSOHN 1959). Additional indications of an effect of quaternary ammonium ions on presynaptic structures can be found in the chapter on previous reports of presynaptic effects of tubocurarine (section 4). We must therefore agree with the statement of RIKER *et al.* (1957) that three possible effects exist for all quaternary ammonium ions: i) a postsynaptic one (depolarizing or stabilizing), ii) an inhibition of the cholinesterase and iii) a presynaptic effect which now, according to previous and present results, obviously can be of either stimulatory or depressive character.

b. *Possible blocking of presynaptic repetitive conduction by tubocurarine*

GRUNDFEST and NACHMANSOHN (1952) and quite recently DETTBARN (1960) have demonstrated a blocking effect of tubocurarine on the axon itself when special methods are used. Relatively high concentrations of tubocurarine are necessary to block the single impulses at Ranvier nodes (10^{-3}M) but it is highly probable that far lower concentrations are capable of producing Wedensky inhibition of conduction without any significant influence of the first impulse in the volley, *i.e.* similar to procaine. Still lower concentrations may — due to a greater permeability of the presynaptic fibres — influence the conduction in this structure in the same way, producing Wedensky inhibition of conduction of tetanic impulses in the last terminals of the motor nerve.

RIKER *et al.* (1957), thus, demonstrated a similar presynaptic effect of small doses of tubocurarine and procain, well below those that interfere with single-volley transmission. The most probable presynaptic mechanism of action of tubocurarine seems, therefore, to be a reinforcement of the naturally occurring tendency to Wedensky inhibition of conduction in the presynaptic fibres demonstrated by KRNJEVIĆ and MILEDI (1958).

Our conclusion as to the presynaptic effect of d-tubocurarine in the neuromuscular transmission of mammals is completely in agreement with results of LAPORTE and LORENTE DE NÓ (1950) on the sympathetic ganglion of turtle. They stated in the summary: "Although tubocurarine produces certain changes in the properties of the ganglion cells curarization is mainly caused by modification in the properties of the presynaptic fibrils, which results in the establishment of blocks of conduction in the presynaptic arborization". These authors

also observed different stages of curarization with a qualitative change of the effects and they also worked with repetitive stimulation to reveal the presynaptic effect.

c. *Competition with ACh for presynaptic receptors?*

BARSTAD (personal communication 1960) has in preliminary experiments demonstrated that ACh itself increases the frequency of the miniature potentials. (See also the conclusion of MASLAND and WIGTON 1940.) The naturally released ACh may possibly have the same effect, an action which, according to recent results (ECCLES *et al.* 1959), indicates a depolarization of the presynaptic terminals, *i.e.* a presynaptic potential. The possibility exists that tubocurarine by means of a competitive mechanism can block this effect of ACh, thereby producing a Wedensky inhibition of the release mechanism. A further discussion of this hypothesis would at the present time be premature and is therefore omitted from this paper.

It is generally accepted that substances which reduce the release of ACh do not produce any Wedensky inhibition. It seems now, however, to be necessary to accept two different mechanisms behind a reduction of the output of ACh, each giving a special picture of inhibition. It is, in this connection, of special interest to mention quite recent results obtained by CHANG (personal communication) with different fractions of *Bulgarus multicinctus* venom. The crude venom has previously been investigated (CHANG 1959 a, b) and a curare-like inhibition of the neuromuscular transmission has been demonstrated. Four different fractions have now been separated, one having a competitive postsynaptic effect similar to that of tubocurarine. Two others have no effect on the receptor substance but inhibit the release of ACh. The surprising result is that these substances, in contrast to botulinum toxin, gave a pronounced Wedensky inhibition. These two fractions seem, in our opinion, to exert a presynaptic effect in its pure form similar to that proposed for tubocurarine in this paper.

Addendum

STRAUGHAN (1960) has recently published results of his investigation on the release of ACh from nerve terminals in the rat and guinea pig phrenic nerve diaphragm preparation. The paper is of special interest in connection with our present results and considerations.

STRAUGHAN demonstrated an astonishing maintainance of the ACh-production throughout prolonged periods of stimulation (20 min). From details in his investigation he drew a similar conclusion as KRNJEVIĆ and MILEDY (1958), previously mentioned in our discussion: "It seems unlikely that there is any depletion of the acetylcholine stores in the terminals during a tetanic period of stimulation even when there is complete neuromuscular block." STRAUGHAN is of the opinion that the decline in the release of ACh during prolonged tetanic stimulation is due to the mechanism demonstrated by the two above mentioned authors, *viz.* an intermittent presynaptic failure of conduction. STRAUGHAN's results indicate that this intermittent presynaptic failure is absent initially during tetanic stimulation, i. e. in the period in which the Wedensky inhibition during curarization takes place. The usually accepted hypothesis of a rapid decline of the ACh-production is not in accordance with STRAUGHAN's results and conclusion. This author, therefore, suggests a rapid reduction of the ACh-sensitivity

(a desensitization) of the motor end-plate as a possible explanation of the Wedensky inhibition. This possibility is, as seen in our discussion, rejected due to results obtained by ourselves and other authors (HUTTER 1952, OTSUKA and ENDO 1960).

STRAUGHAN also mentions another possible explanation for the Wedensky inhibition "an enhancement of the blocking activity of curare (CHOU, 1947)". CHOU demonstrated that "the effect produced by tubocurarine is greater when the muscle is working and is a function of the rate of stimulation".

The Wedensky inhibition seen during curarization is also a phenomenon characterized by a rapid increase of the block of transmission of impulses of higher frequencies. A pure description of the phenomenon, as found in CHOU's work, cannot give any explanation of the mechanism behind either the decline of the single contractions or the Wedensky inhibition. We are of the opinion that both the well known fact described by CHOU and the Wedensky inhibition are due to the same mechanism, and have in our main paper tried to find a satisfactory explanation, which also fits well with the new results obtained in STRAUGHAN's thoroughly performed investigation of the ACh-production during tetanic stimulation.

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Effect of Reserpine on the Release of Catecholamines from Isolated Nerve and Chromaffin Cell Granules

By

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Abstract

Euler, U. S. v. and F. Lishajko. *Effect of reserpine on the release of catecholamines from isolated nerve and chromaffin cell granules.* Acta physiol. scand. 1961. *52.* 137—145. — In a suspension of bovine splenic nerve granules, reserpine in concentrations of 10^{-8}—10^{-5} M strongly inhibits the spontaneous release of noradrenaline. The catecholamine releasing action of tyramine on nerve granules is unaffected by low concentrations of reserpine, but is almost completely blocked by concentrations of 10^{-5} M. Reserpine is less active as inhibitor on suprarenal medullary granules from the rabbit and still less on cats' medullary granules. The spontaneous release occurs at a lower rate in rabbit's medullary granules than in those from the cat. No difference in the rate of release was found between adrenaline and noradrenaline from the cat's medullary granules. After administration of dopa 100 mg/kg *i.v.* in the rabbit the rate of release from the medullary granules was greatly enhanced. The possible action of reserpine as enzyme inhibitor is briefly discussed.

In a previous paper by Euler and Lishajko (1960 b) it was reported that noradrenaline was released at an increased rate from isolated and resuspended bovine splenic nerve granules on incubation with reserpine. The concentrations necessary to cause a marked release were high, however; thus about 0.5 mM (0.3 mg/ml) reserpine (as phosphate) per ml of suspension fluid was required to cause about 90 per cent release as compared with 30—40 per cent "spontaneous" release during 30 min at + 20° C. In concentrations below 0.1 mM no releasing effect was observed. Similar effects were obtained with granules from the heart of two cyclostomes, *Myxine glutinosa* and *Petromyzon fluviatilis* (Östlund *et al.* 1960).

Subsequent experiments with a slightly modified technique have revealed an effect of reserpine of a different kind. It was thus observed that incubation of catecholamine granules from nerves with reserpine in low concentrations to a large extent prevented the release of noradrenaline. In the present report are described some results obtained with granules from nerves and suprarenal chromaffin cells on incubation with reserpine.

Method

Catecholamine containing granules were prepared from bovine splenic nerves by the method described previously (EULER and LISHAJKO 1961 a) based on squeezing the carefully dissected nerve trunks between nylon cylinders in the cold and uptake of the press juice in 0.13 m K-phosphate at pH 6—7.

While in the previous study the sediment was resuspended in K-phosphate, the suspension was used directly in the present experiments, after removal of larger particles by centrifugation at 1,000 *g* for 5 min at 0—4° C. Almost no spontaneous release of noradrenaline occurred during 5 hours when the suspension was kept at a temperature of + 4° C at pH 6—7. Lyophilized reserpine phosphate (kindly supplied by Dr. A. Plummer, Ciba, Summit, N. J.) was added in aqueous solution. To the controls was added the same quantity of redistilled water.

After addition of reserpine the suspension was incubated for 2 hours in a water bath at + 20° C. One sample was kept at + 4° C to serve as an absolute control. One or two aliquots of the original suspension were incubated without reserpine in order to measure the spontaneous release.

In a number of experiments tyramine hydrochloride was added to the incubation fluid.

At the end of the incubation period the samples were centrifuged for 30 min at 50,000 × *g* at + 4° C and the sediment extracted with hydrochloric acid and metaphosphoric acid added according to the method of EULER and LISHAJKO (1961 a). After centrifugation and removal of the sediment the noradrenaline was estimated fluorimetrically according to the method of EULER and LISHAJKO (1961 b).

In special tests it was established that reserpine does not interfere with the fluorimetric estimation of noradrenaline in amounts up to 250 μg which are considerably higher than those occurring in the samples of the present study. Similar controls were made with tyramine.

Granules were also prepared from the suprarenal glands of the rabbit and the cat in the same way as described above.

Results

1. Splenic nerve granules.

a. *Reserpine.*

Fig. 1 shows the effect of incubation of splenic nerve granules with different concentrations of reserpine. As seen in the figure reserpine causes a very pronounced inhibition of the release in concentrations of 1 to 100 μg per ml. Even in a concentration as low as 0.1 μg/ml a marked inhibition of the release is observed. By pretreatment for 30 min at + 4° C the inhibitory action of reserpine is observed even in a concentration of 0.01 μg per ml or 1.6×10^{-8} M.

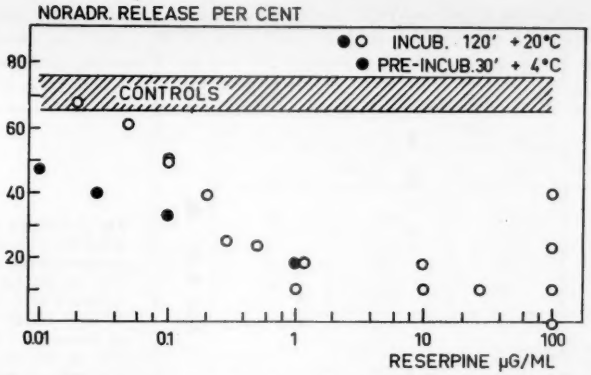

Fig. 1. Inhibitory effect of reserpine on the release of noradrenaline from a suspension of isolated bovine splenic nerve granules. Controls, spontaneous release. Empty circles: per cent NA release after 2 hours incubation at + 20° C, pH 6—7. Filled circles: Same, with pre-incubation for 30 min at + 4° C.

No difference in the degree of inhibition has been noticed when the pH was varied between 6 and 7 in the incubated suspension, if reserpine was present in concentrations of 10 µg/ml or lower. In higher concentrations pH 6 was used in order to prevent precipitation of reserpine.

b. *Reserpine and tyramine.*

It has been shown previously that tyramine can cause a release of noradrenaline from isolated granules (SCHÜMANN 1960, EULER and LISHAJKO 1960 a). It therefore seemed of interest to study whether the releasing effect of tyramine was influenced by reserpine in concentrations which inhibit the release. As standard concentration of tyramine 10 µg/ml (7.3×10^{-5} M) has been chosen since this consistently has shown good releasing effect.

Fig. 2 shows the time course of the effect of tyramine alone on the release of noradrenaline from a suspension of splenic nerve granules in comparison with controls. Since it was shown that tyramine is partly inactivated by monoamineoxidase present in a resuspension of the sediment (W. Hardegg, personal communication) a second dose of tyramine was added after 1 hour in later experiments. The inactivation of tyramine is possibly reflected in a slight deviation of the release curve as seen in Fig. 2.

Fig. 3 demonstrates that the increased release of noradrenaline caused by tyramine is abolished in the presence of reserpine in a concentration of 10 µg/ml. In very low concentrations, however, 0.01—0.03 µg per ml, reserpine, though still inhibiting the "spontaneous" release, does not block the action of tyramine. In the intermediary range of concentrations, 0.1—1 µg/ml, reserpine exerts a gradually increasing blocking effect on the tyramine action. In these

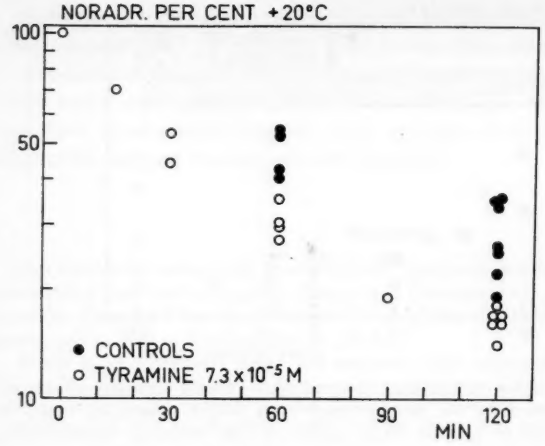

Fig. 2. Release of noradrenaline from a suspension of bovine splenic nerve granules at pH 7.0 during incubation at + 20° C. Spontaneous release and effect of tyramine 7.3×10^{-5} M (10 µg per ml). Ordinate: per cent noradrenaline in sediment after incubation.

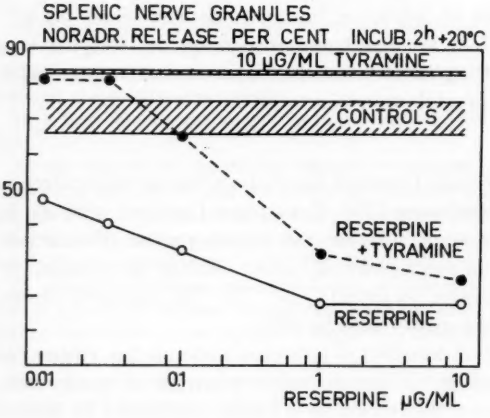

Fig. 3. Effect of reserpine on the noradrenaline releasing action of tyramine on isolated bovine splenic nerve granules. Incubation for 30 min at + 4° C followed by 2 hours at + 20° C. Effect of tyramine alone and control release at + 20° C for 2 hours indicated. Tyramine 7.3×10^{-5} M in all experiments, reserpine concentration on the abscissa.

experiments the granule suspension was pre-incubated with reserpine for 30 min at + 4° C which did not influence the noradrenaline content of the granules. The controls were treated in the same way. From these experiments it is evident that reserpine in concentrations of the same order as those of tyramine almost completely inhibits the effect of tyramine. This appears of interest since tyramine is assumed to act by substituting noradrenaline in the granules on account of its stronger basic properties (SCHÜMANN, personal communication).

Table I. Release of adrenaline (A) and noradrenaline (NA) after incubation of cat suprarenal medullary granules for 2h at + 20° C, pH 7.0 with reserpine and tyramine in per cent of amount present in a suspension incubated 2h at + 4° C. Proportion A: NA in sediment of original suspension, 1: 2.5 and 1:0.97 respectively. Expt. I and II

	A		NA	
	I	II	I	II
Control	75	89	77	89
Reserpine 1.6×10^{-5} M	69	79	75	86
Tyramine 7.3×10^{-5} M	85	94	86	95
Reserpine + tyramine	78	92	84	94

II. Suprarenal medullary granules.

A series of experiments were also made with granules from the suprarenal medulla of the cat and the rabbit.

a. *Cat.*

The suprarenals were removed under nembutal anesthesia and granules prepared. The suspension of granules was incubated with reserpine and tyramine as in previous experiments. Table I shows the effect of incubation on the release of adrenaline and noradrenaline from the granules.

As seen in the table reserpine has a very slight inhibitory effect on the "spontaneous" release as compared with the action on splenic nerve granules. Tyramine has a moderate releasing effect on noradrenaline as well as adrenaline, which is in keeping with the results reported by SCHÜMANN (1960) for bovine medullary granules. Even in the presence of reserpine 1.6×10^{-5} M, tyramine has almost the same releasing effect as without reserpine, in marked contrast to the effects observed with nerve granules (Fig. 4).

b. *Rabbit.*

The rabbits were killed by a blow on the neck and medullary granules prepared in a similar way as for the cat. The inhibitory effect of reserpine was somewhat more marked than on the cat's medullary granules. In Table II are given the results of incubation experiments of the same kind as in Table I with granules from untreated animals and after administration of dopa. Dopa was given *i.v.* in a dose of 100 mg per kg after dissolution in Serpasil® solvent (1 ml for 100 mg) and dilution in 15—20 ml saline under warming to about 40° C. Figures are only given for adrenaline since the relative amounts of noradrenaline were small.

In spite of the fairly large variations between the single experiments it can be seen from Table II that reserpine exerts an inhibitory action on the release of adrenaline in 5 of the 6 experiments. Tyramine increased the release likewise

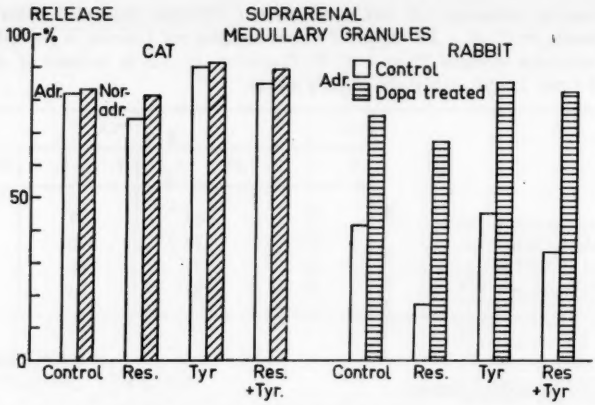

Fig. 4. Release of adrenaline and noradrenaline (cat, left) and adrenaline (rabbit, right) after incubation of medullary granules for 2 hours at + 20° C, pH 7.0 with reserpine and tyramine, in per cent of amount present in a suspension incubated 2 hours at + 4° C. Bars with horisontal stripes (right) indicate actions on medullary granules after pretreatment of rabbit with dopa 100 mg/kg.

Table II. Release of adrenaline after incubation of rabbit suprarenal medullary granules for 2 h at + 20° C, pH 7.0 with reserpine and tyramine in per cent of the amount present in a suspension incubated 2 h at + 4° C. Expt. I—VI

	Untreated			Average	1 h after 100 mg/kg dopa i.v.			Average
	I	II	III		IV	V	VI	
Control	26	37	60	41	74	65	87	75
Reserpine 1.6×10^{-5} M ..	11	22	17	17	74	54	74	67
Tyramine 7.3×10^{-5} M ..	37	45	54	45	83	81	91	85
Reserpine + tyramine	33	49	18	33	76	82	87	82

in 5 of 6 experiments. On the other hand reserpine was relatively inefficient in preventing the releasing effect of tyramine (Fig. 4). Using a high concentration, 200 μg/ml of reserpine, a releasing effect was noted, however, being about twice as strong as the spontaneous release at + 20° C.

Discussion

While the "spontaneous" release of noradrenaline from bovine splenic nerve granules did not differ markedly from that in cat's medullary granules, both were considerable higher than the release from rabbit's medullary granules.

These behaved in this respect more like the bovine medullary granules (HILLARP and NILSON 1954).

With regard to the effect of reserpine on the release of catecholamines from storage granules of various origins the picture is complicated. In resuspended granules from bovine splenic nerves it was shown previously (EULER and LISHAJKO 1960 b) that reserpine increased the rate of release in concentrations of about 0.1 mg/ml and higher. This finding has been confirmed for granules both from nerves and from chromaffin cells in later experiments, but it was also found that when reserpine was added in lower concentrations directly to the suspension of splenic nerve granules and the mixture incubated at + 20° C for various times it had an opposite effect which was very marked. Thus an almost complete inhibition of the release was observed with reserpine in concentrations down to 1 μg/ml or 1.6×10^{-6} M. A definite inhibitory effect was noticed even in a concentration of 1.6×10^{-8} M if the granule suspension was preincubated with reserpine at low temperature.

This oligodynamic effect of reserpine also modifies the releasing action of tyramine. While it will not prevent the tyramine action in concentrations of about 5×10^{-8} M, its inhibitory action of the tyramine releasing effect increases with higher concentrations. Thus the tyramine releasing action is almost completely blocked when reserpine is present in a concentration of 1.6×10^{-5} M. This action is difficult to explain on the basis of the assumption that tyramine simply substitutes noradrenaline in the granules on account of its stronger basicity. No explanation can at present be given for the striking effect of reserpine in low concentrations on the noradrenaline release on incubation of splenic nerve granules, but it appears not unplausible that reserpine selectively blocks some enzymatic system involved in the release. Since reserpine is known to cause a depletion of the catecholamine stores in nerves and organs it may be assumed that it also blocks the mechanism by which storage is effected (CARLSSON et al. 1957, BERTLER 1961). A similar effect has been postulated for bretylium and guanethidine by ZAIMIS (1960). The depletion of organs may be explained by a continuous leakage of the transmitter from the stores, not followed by repletion. Recently MUSCHOLL (1960) has shown that infused noradrenaline is not taken up by the hearts of reserpine-treated rats. It is also interesting to note that SANO et al. (1960) found a blocking effect of reserpine on the uptake of 5-HT or adrenaline by thrombocytes.

In this context it should be recalled that ABOOD and ROMANCHEK (1956—57) observed a marked inhibition of oxidative phosphorylation in brain mitochondria by reserpine in a concentration of 2×10^{-5} M.

The inhibitory effect of reserpine on the release of catecholamines is less marked on suprarenal medullary granules of the rabbit and very slight on such granules from the cat. STJÄRNE and SCHAPIRO (1959) observed no effect of reserpine in concentrations of 0.05—5 μg/ml on isolated bovine medullary

granules. Using 200 μg/ml we have observed a marked releasing effect on the rabbit medullary granules, however. This effect is in keeping with the direct releasing action of reserpine when given intravenously in large amounts (MUSCHOLL and VOGT 1957). Similarly the blocking effect of reserpine on the releasing effect of tyramine is weaker than in the case of splenic nerve granules. This is particularly obvious after pretreatment of the rabbit with dopa which greatly enhances the spontaneous release of adrenaline from isolated medullary granules on incubation at + 20° C. It is also worth mentioning that no difference was observed between the release from noradrenaline and adrenaline storing granules in the cat's medulla.

The quantitative rather than qualitative difference in action of reserpine on the catecholamine storage granules from different tissues may be correlated with the differences reported between the depleting effect of reserpine on different organs and in different species. Thus the cat's suprarenal medulla is known to respond less readily to reserpine.

The reason why resuspended splenic nerve granules should tend to react with increased rate of release for reserpine in concentrations which have an inhibitory effect on the original suspension is not clear, but may be due to the loss of some factor or factors in the resuspended preparation. Even in concentrations of 0.2 mg/ml, reserpine did not enhance the release of noradrenaline when added directly to the original suspension of nerve granules.

The strong enhancement of the spontaneous release in rabbit's medullary granules after pretreatment of the animal with dopa seems to indicate an important disturbance in the granules. It appears less probable that this effect is due to the formation of dopamine since this does not influence the release (SCHÜMANN 1960, EULER and LISHAJKO 1960 a) from granules either from nerves or from medullary cells.

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The Effect of Choline 2,6-Dichloro Phenyl Ether Bromide (TM-25) on Organ Content and Urinary Excretion of Catecholamines

By

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Abstract

SCHAPIRO, S. *The effect of choline 2,6-dichloro phenyl ether bromide (TM-25) on organ content and urinary excretion of catecholamines.* Acta physiol. scand. 1961. *52.* 146—149. — Daily injections of choline 2,6-dichloro phenyl ether bromide (TM-25) in the rat greatly reduces the urinary output of noradrenaline and to a lesser extent that of adrenaline. After 5—6 days of treatment with TM-25 the catecholamine content of adrenals, heart and spleen is considerably reduced. It is inferred from the results that TM-25 interferes with the production, release or storage of catechol amines in the adrenergic nerves and possibly in the adrenals.

Several contributions have been made during recent years towards elucidating the action pattern of the choline phenyl ethers. As a result of these studies it has been demonstrated that some compounds in this group such as TM-6 (choline-p-tolyl ether bromide) and TM-10 (choline-2,6-xylyl ether bromide) act as inhibitors of amine oxidase in vitro (BROWN and HEY 1952, 1956, GRIESEMER and WELLS 1956) though not in vivo (CORNE and GRAHAM 1957). The catecholamine content of organs does not seem to have been studied after administration of TM-6, but it has been reported that it does not alter the catecholamine excretion in urine in the cat (CORNE and GRAHAM 1957). TM-10 on the other hand, blocks adrenergic nerve action (EXLEY 1956, 1957, BAIN and FIELDEN 1956). It has a slight depleting effect on the content of catecholamines in the suprarenals of rats when administered daily over 2 weeks (COUPLAND and EXLEY 1957). The urinary excretion of catecholamines as a convenient

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Table I. Effect of intraperitoneal injection of 4 mg/kg TM-25 on organ catechols (μ/g) in rats

	Controls 10 animals		Group A 6 days TM-25 5 animals		Group B 5 days TM-25 (after 7 daily inj. of saline) 5 animals		Group C One week after end of treatment with TM-25 5 animals	
	NA	A	NA	A	NA	A	NA	A
Spleen	0.96	—	0.21	—	0.13	—	0.42	—
Heart	0.62	—	0.43	—	—	—	0.46	—
Adrenals	280	900	79.5	560	56	300	140	864

relative measure of the rate of release, does not seem to have been investigated. No depletion of the organ content of catecholamines was observed in short-term experiments on guinea-pigs (EULER and LISHAJKO, personal communication).

In view of the marked effects induced by small changes in the composition of these compounds it seemed of interest to study the organ content and urinary excretion of catecholamines after administration of TM-25, the dichloro analogue of TM-10, which was kindly placed at our disposal by Dr. W. A. BAIN.

Methods

Five female rats (weight 222—310 g) were placed in a community cage and TM-25 was injected daily for 6 days intraperitoneally into each animal in a dose of 4 mg per kg in 0.5 ml saline. After this period the rats were sacrificed by a blow on the head, and adrenals, spleens and hearts analyzed for catechols by the fluorimetric technique of EULER and FLODING (1955) after mincing and extraction with 5 per cent trichloroacetic acid (Group A).

In another series 10 female rats were placed in a community metabolism cage and injected with 0.5 ml saline daily *i. p.* for 7 days. Urine was collected daily during this control period. TM-25 was then injected daily in the same dose as in group A for 5 days. Five rats were then sacrificed as previously and organs analyzed for catechols (Group B). The remaining 5 rats received saline for an additional 7 day period and were then sacrificed as before (Group C). Urine was saved over the first 5 days and analyzed for catechols. Adrenals, hearts and spleens were also analyzed for catechols as described above. Control values for normal organ catechols were obtained from 10 normal rats which were sacrificed by a blow on the head.

Results

1. *Effect of TM-25 on urinary catechols.* In Fig. 1 is shown the catechol excretion values in ng/kg/hr before, during and after treatment with TM-25. As seen in the figure the drug drastically reduced the output of both adrenaline and noradrenaline .Following cessation of treatment the adrenaline remained low for several days, while noradrenaline tended to return to control levels.

2. *Effect of TM-25 on organ catechols.* After treatment for 5 and 6 days with

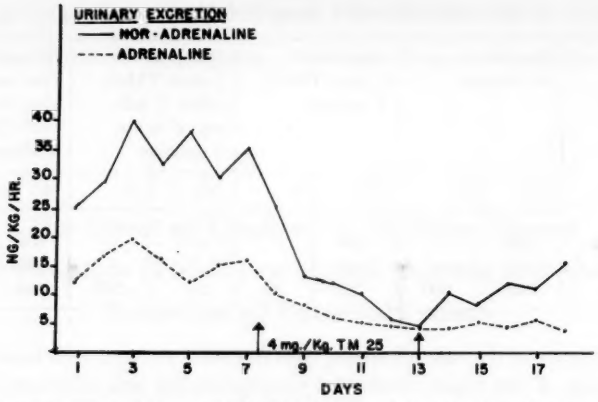

Fig 1. Urinary catechols before (10 animals), during (10 animals) and after (5 animals) daily administration of TM-25 for 7 days.

TM-25 the catechol content of the spleen and adrenals was reduced to approximately 1/5—1/2 of the resting level (Table I). The heart apparently was more resistant to the depleting effects. After one week without treatment, organ catechols were returning to control levels, although the normal figures were not quite reached.

Discussion

The results reported here have shown that TM-25, like TM-10 (COUPLAND and EXLEY 1957) diminishes the catechol content of the adrenals in the rat. We have further observed that the spleen loses most of its catecholamines when these were examined at the end of 5—6 days treatment with TM-25. The fall in catecholamines in the heart was less marked, however. Simultaneously with these organ changes, the urinary excretion of noradrenaline and adrenaline is decreased. As the organ contents are partially restored to normal levels 7 days after cessation of treatment, the urinary catechols also tend to return to normal values.

It has been recently shown (SCHAPIRO 1958) that a catecholamine blocking agent, dibenzyline, in a dose of 10 mg/kg, causes an increased urinary excretion of noradrenaline in rats and depleted various organs of their catechols. BENFEY, MAZURKIEWICZ and MELVILLE (1958) also showed that sympathetic blocking drugs increased the fraction recovered in urine after injection of adrenaline and noradrenaline. It is indeed possible that these blocking agents compete for metabolic receptor sites (as contrasted with effector receptor sites) with a resultant "overflow" of catechols into the blood and subsequently into the urine, in a way analogous to that described in its first step by BROWN and GILLESPIE (1957).

The present results are obviously in contrast to the effect of the sympatholytic agents, although TM-25 has been shown to have a short-lasting adrenolytic action (McLEAN *et al.* 1960). If the TM compounds acted only as inhibitors of amine oxidase and blocked liver inactivation of adrenaline (GRIESEMER and WELLS 1956) one would expect an increased urinary excretion of catechols. The decreased output actually found agrees with the suggestion of COUPLAND and EXLEY that in vivo an inhibition of catechol synthesis may actually occur in addition to the blocking of the release. This action seems to be on the adrenals as well as on the adrenergic nerves although the more marked fall in the noradrenaline excretion in urine suggests that the greater part of the effect is on the adrenergic nerves. This is further supported by the demonstration that TM-10 prevents the transformation of dopamine to noradrenaline in human chromaffin tissue (BAIN and FIELDEN 1957).

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Taste Functions in the Carp

An Electrophysiological Study on Gustatory Fibres

By

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Abstract

J. Konishi and Y. Zotterman. *Taste functions in the carp. An electrophysiological study on gustatory fibres.* Acta physiol. scand. 1961. *52.* 150—161. — The electrical responses of the taste fibres in carp (*Cyprinus carpio* (L.)) were recorded during the application of various sapid substances. A comparison of the integrated electrical responses from the glossopharyngeal nerve innervating the palatal organ, the facial nerve innervating the lining of the mouth and the barbels, and the branchial nerve innervating the gill rakers showed that the palatal organ seems to be the principal taste organ of this fish. The records from the glossopharyngeal nerve revealed that this nerve contains chemoceptive fibres responding to a great variety of sapid solutions. The response to acid and to sucrose was particularly strong, while the integrated response to 0.5 M NaCl always was considerably weaker. The application of water to the palatal organ had no stimulating effect. Especially noteworthy was a generally quite strong response to the application of human saliva.

A study of the records from 114 single taste fibres from the glossopharyngeal nerve revealed that the fibres could be divided into seven groups according to their response pattern. Highly specific response to NaCl was encountered in 14 fibres. Acetic acid (0.005 M) seemed to stimulate all taste fibres, except salt fibres. Specific response to weak acid was noticed in 3 fibres. The fibres which responded to human saliva were also stimulated by sucrose (except for 3 fibres). Touching the surface of the palatal organ did not stimulate any taste fibres.

In fishes receptor structures resembling taste buds in mammals have been described by many anatomists. These receptors are situated around the mouth, in the oral cavity and on the gill rakers, as well as in barbels of various fishes. They are found particularly densely in the roof of the mouth of the carp, in its

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palatal organ. This organ is supplied by nerve fibres from the glossopharyngeal nerve. HOAGLAND (1933) first recorded electrical responses from the facial nerve of the catfish in response to various sapid solutions applied on the barbels. In this research we have tried to obtain more detailed data about the gustatory mechanisms of a fresh water fish.

Methods

A total of 78 carps (*Cyprinus carpio* (L.)), with an average body length of 28 cm were used in this study. The fish was decapitated and the vagal lobe as well as the medulla oblongata were destroyed. Integrated records in response to sapid solutions were obtained from bundles of the glossopharyngeal nerve, from the facial nerves innervating the linings of the mouth, and from the nerve bundles innervating the gill rakers. In order to expose the bundles of the glossopharyngeal nerve, which innervate the palatal organ, this organ was carefully separated from the palatal cartilage and lifted by hooks. In all experiments we used the bundle innervating the anterio-lateral part of the palatal organ, running closely to the anterior margin of the palatal cartilage, because this bundle offered the longest stretch of nerve (available length for recording is about 5 mm). The cartilage was removed in order to facilitate further dissection. The nerve was lifted on to a platinum electrode attached to a micromanipulator and connected to the input of an RC coupled amplifier. The other input was attached to the tissues and grounded. Responses of the entire nerve were recorded, using an electronic integrating device which has been described in earlier papers.

To make single fibre preparations, the nerve was placed on a small platform with a black surface offering a good contrast to the nerve strands. The epineural sheath was then dissected off and the nerve split into small bundles, from which fine strands were separated by further dissection under a binocular microscope. Under Ringer's solution, the entire glossopharyngeal nerve preparations could be kept responsive for four hours or more. The facial nerve was exposed from the orbit. The recording from the branchial nerve was done in isolated preparation.

The test solutions were applied by a specially made apparatus incorporating a signalling device. The taste solutions mainly tested were sodium chloride (0.5 M), sucrose (0.5 M), quinine hydrochloride (0.01 M) and acetic acid (0.005 M, pH 3.8) in aqueous solutions. Some fibres were also tested with solutions of saccharine, glycerol (0.5 M) and ethylene glycol (0.5 M).

Results

Integrated response

Glossopharyngeal nerve

Water hardly stimulates any of the chemoreceptors of this fish, and especially after a previous rinse with water. The palatal nerve responded to all four conventional classes of taste substances: salt, sucrose, quinine and acid; especially strongly to acid and sucrose (Fig. 1). In all the nerve preparations tested, glycol and glycerol also elicited a positive response, while saccharine gave a very feeble response if any. The responses to repeated application of a test solution decreased, even if interspersed with water rinses. It is particularly notable that human saliva had a strong effect on the chemoceptors, and gave a much bigger integrated response than the 0.5 M NaCl and 0.01 M quinine solutions. The

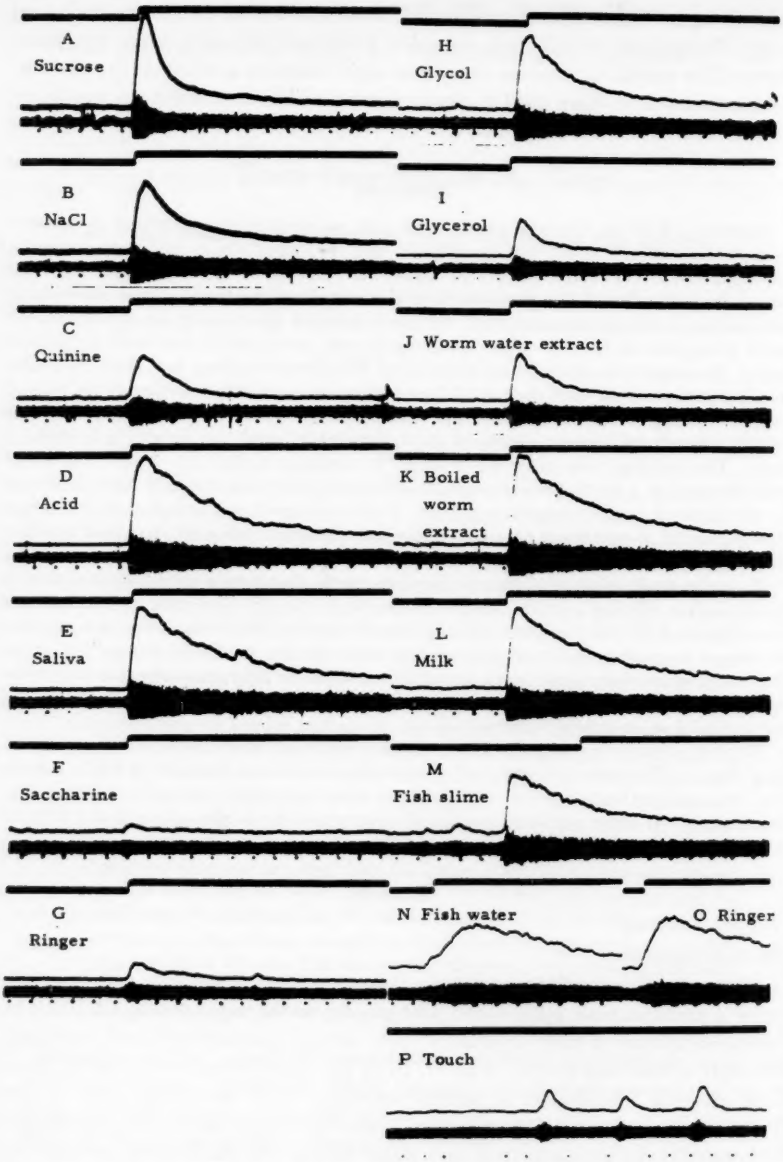

Fig. 1. Electrical responses from the entire glossopharyngeal nerve of carp to the application of various solutions to the palatal organ and to touch. In each tracing are recorded, from top to bottom: the signal showing the moment of application, the integrated response, and the direct spike response. Records N and O were recorded by high amplification. Time in sec.

response to fish Ringer's solution was quite small, even after a previous rinse with water. Water extract of earth worm also produced a positive response, and a boiled extract was particularly effective. Milk (1 % dry milk in distilled water) also produced a massive response. The mucous substances covering the body surface of the fish gave a positive response. It is a surprising fact that the chemoceptors responded, though in small degree, even to the water in which fish had been kept (Fig. 1, N). The response to dilute solutions as well as to the water the fish had been kept in was usually found to rise rather slowly to attain a slowly declining plateau. It is interesting to note that the responses to sugar were comparatively phasic in both integrated and direct spike responses, in spite of large magnitude, while those to saliva as well as to worm extract and milk lasted much longer.

Besides chemoceptive and motor fibres the glossopharyngeal nerves contain fibres which respond very selectively to mechanical stimulation, as stated below. The response to touch is, however, much smaller in magnitude than that to chemical stimulation (Fig. 1, P). The most sensitive area to touch seemed to be localized in the lateral sides of the palatal organ.

Facial nerve

The response to sapid solutions of the facial nerve in this fish was quite small, as in Fig. 2, I. On the other hand, tactile stimulation caused relatively large response. There is no doubt, however, that all taste substances which stimulated the palatal organ also were able to produce positive responses in the facial nerve, although to a minor degree. This smaller sensitivity might be attributed to the small amount of chemoreceptors located around the mouth. A smaller sensitivity of this nerve in response to sapid substances was also reported by HOAGLAND (1933) in catfish.

Branchial nerve

Typical records of the response from the branchial nerve innervating the chemoreceptors on gill rakers are reproduced in Fig. 2, II. All sapid substances which stimulated the palatal organ produced positive responses also in this nerve. Similarly to the glossopharyngeal nerve, the most effective stimulant was sugar. Of the two peaks in the record A in Fig. 2, II, the later one was caused by the sugar solution flowing over those parts of the gill raker which were not stimulated when the solution was first poured out from pipette. Touch stimulation applied to the gill rakers produced a response with similar height as did chemical stimulation.

Nerve innervating the ventral fin of the bullhead (Cottus quadricornis L.)

Before testing, we have thought that the narrow ventral fins of this species may be capable of mediating chemical perception. It was found, however, that the fins did not respond to any sapid substances at all, not even to acid, but served as very sensitive tactile organs (Fig. 2, III).

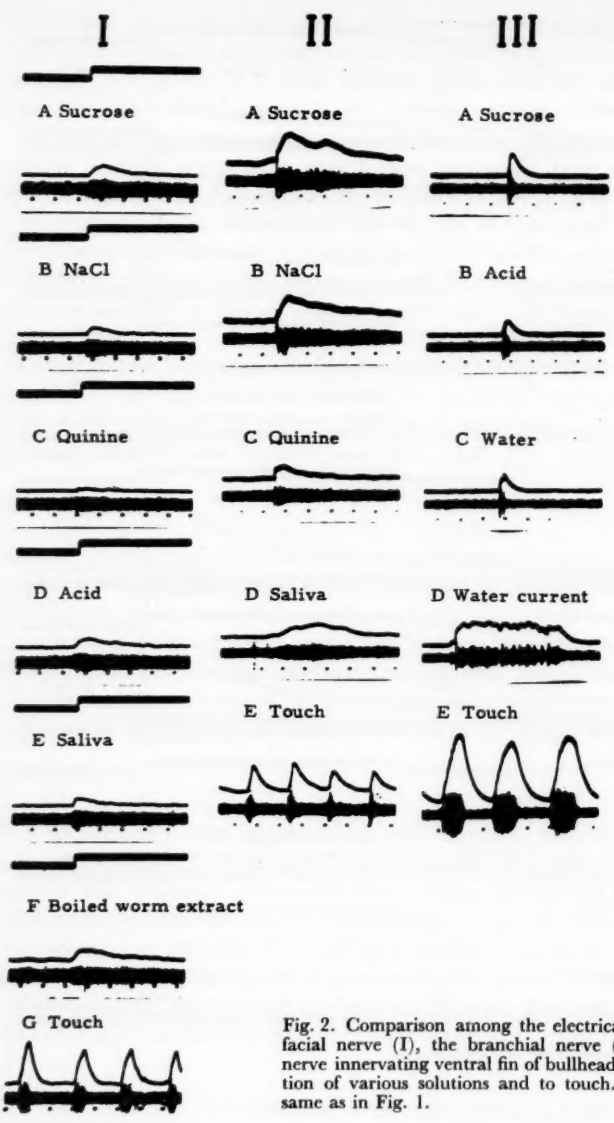

Fig. 2. Comparison among the electrical responses from the facial nerve (I), the branchial nerve (II) of carp, and the nerve innervating ventral fin of bullhead (III) to the application of various solutions and to touch. Amplification is the same as in Fig. 1.

Single fibre analysis

General

From the experiments on fine strands of the glossopharyngeal nerve containing several active fibres, two types of fibre were distinguished: One fibre type showed

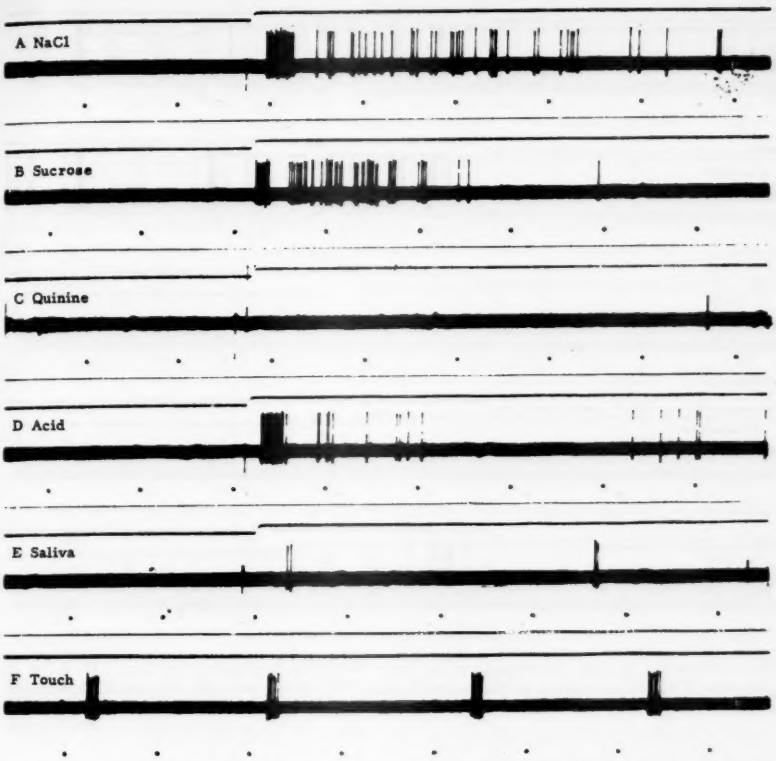

Fig. 3. Response of single glossopharyngeal nerve fibre classified under the Type I (b) to the application of various solutions to the palatal organ. Record F, responses of single touch fibre in the glossopharyngeal nerve to the tactile stimulations. A signal shows the moment of application of the test solution. Time in sec.

phasic activity and the other gave persistent activity. In addition, it was often observed that some fibres did not display a continuous discharge, but showed grouped discharges as in Fig. 4.

None of the taste fibres tested responded to tactile stimulation to the palatal organ. The number of touch fibres seems to be relatively small compared with the chemoceptive fibres. HOAGLAND (1933) demonstrated in the catfish that the amplitude of the spikes from the facial nerve caused by touch is much larger than that of taste fibres, and this finding was confirmed by the present experiments on the facial nerve in the carp. Such a difference in spike height between touch and taste fibres could, however, not be observed in the glossopharyngeal nerve of the carp (Fig. 3, F). To discharge the touch fibres of the glossopha-

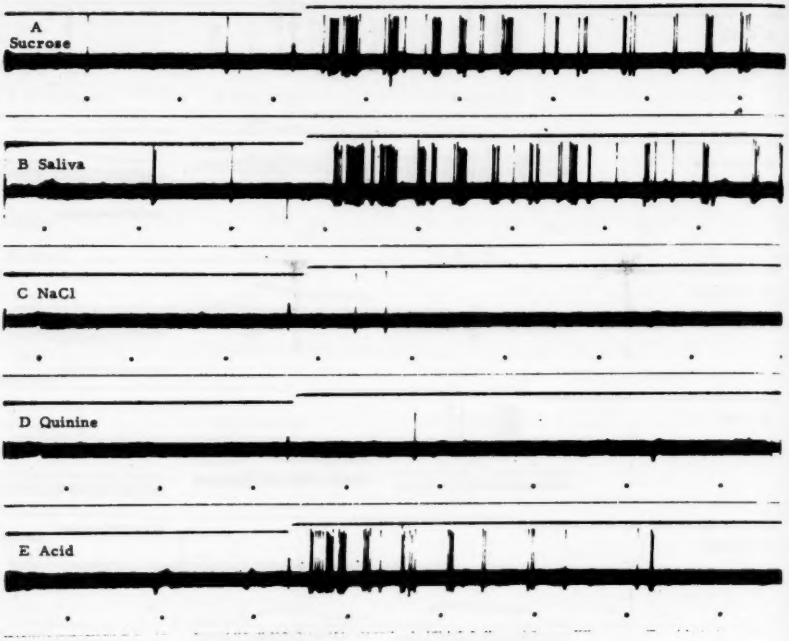

Fig. 4. Response of single glossopharyngeal nerve fibre classified under the Type III to the application of various solutions to the palatal organ. This is an example of grouped discharges. Time in sec.

ryngeal nerve, a stronger mechanical stimulation was needed than the flow of sapid solution over the palatal organ.

According to their response pattern, individual palatal chemoceptive fibres seem to be divided into seven groups as will be seen from Table I. Among these different fibres the Types I, II and III are the most common. Weak acid stimulated all types of taste fibres, except the majority of the salt fibres. Acetic acid in concentrations above 0.01 M (pH 3.2) was found to impair the sensitivity of chemoceptors to all kind of taste substances, or to cause a temporary lowering of the sensitivity.

Type I

This is the fibre type which responds to many taste substances (NaCl, sugar, acid, glycerol and glycol), and even to human saliva, but not to quinine (Fig. 3). Out of 37 such preparations, only 6 did not respond to saliva.

Type II

All kind of taste substances used, stimulated the fibres of this type, but to

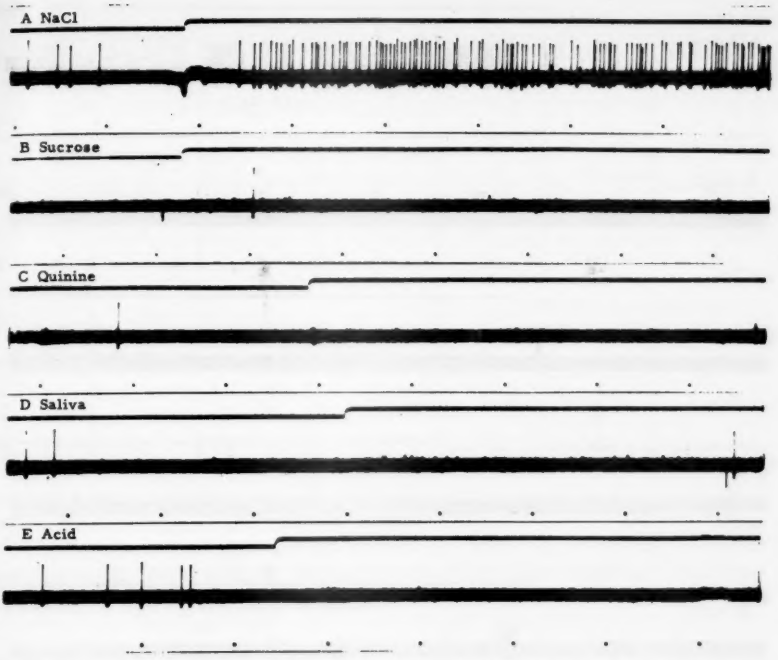

Fig. 5. Response of single glossopharyngeal nerve fibre classified under the Type IV to the application of various solutions to the palatal organ. Note the specific response to NaCl, and no response even to acid. Time in sec.

different degrees showing a great variety of individual response. In contrast to Type I, the Type II fibres responded positively to quinine. Type I and II are the most unspecific taste fibres in the glossopharyngeal nerve of the carp.

Type III

This fibre type is characterized by its high sensitivity to sweet tasting substances, and was very frequently found in the palatal nerve (Fig. 4). It also responded to acid but did not respond to salt and quinine. Glycerol and glycol, which taste sweet to humans, produced positive responses in this fibre. Glycol was always more effective than glycerol, contrary to the results of behavioural studies reported by TRUDEL (1929), showing no response to glycol in his fishes, though this might be due to the difference between species. Saccharine seems to have very little effect. It is particularly remarkable that the human saliva selectively stimulated these sweet fibres quite as strongly as sucrose, and in some cases even more so. Out of the 21 preparations, saliva failed to stimulate this fibre type in only two exceptional cases. As will be stated below, the fibres of

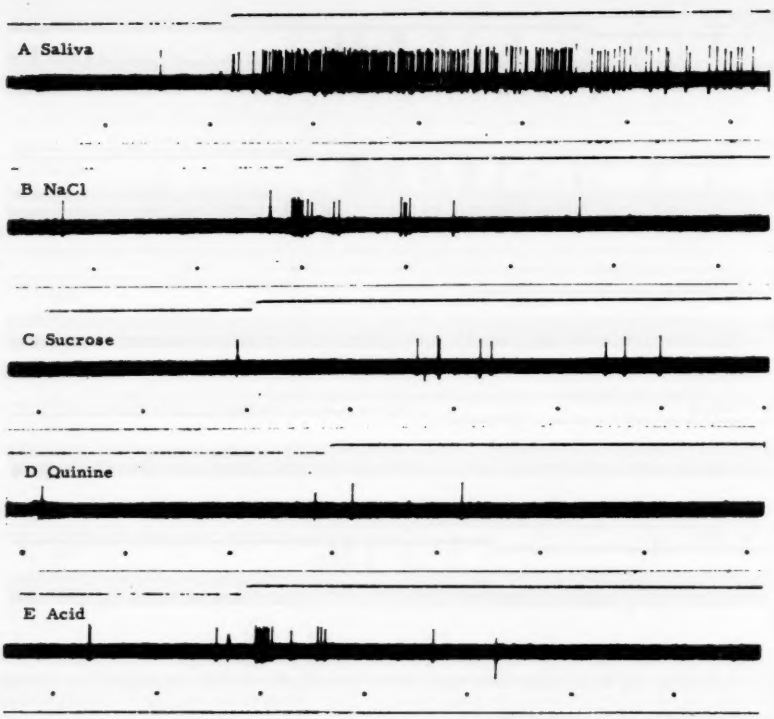

Fig. 6. Response of single glossopharyngeal nerve fibre classified under the Type VI to the application of various solutions to the palatal organ. Time in sec.

the Type IV, V and VII were never stimulated by saliva. On one occasion, the fibre responded slightly to salt in addition to sucrose, saliva and acid. The stimulating effects of the latter were, however, by far stronger than that of the former. On only one occasion, a functional single fibre was found which responded to sugar as well as to quinine.

Type IV

The fibres classified under this type responded primarily to salt solutions (Fig. 5). It is interesting to note that among the 16 fibres of this type so far studied, all except two were not stimulated by acid at the concentration used. This is the only taste fibre type which was insensitive to weak acid.

Type V

These fibres responded both to quinine and acid and in this respect resemble some of the monkeys' fibres (GORDON *et al.* 1959). The number of such fibres seems to be relatively low.

Table I

Stimulus / Fibre Type	NaCl (0.5 M)	Acetic acid (0.005 M)	Quinine (0.01 M)	Sucrose (0.5 M)	Saliva	Number of preparations
I a.....	+	+	−	+	+	31
b.....	+	+	−	+	−	6
II.....	+	+	+	+	+	21
III a.....	−	+	−	+	+	19
b.....	−	+	−	+	−	2
IV a.....	+	−	−	−	−	14
b.....	+	+	−	−	−	2
V.....	−	+	+	−	−	13
VI	−	(+)	−	−	+	3
VII	−	+	−	−	−	3

Type VI

Fibres which, surprisingly enough, showed a vigorous response only to saliva were but rarely found (Fig. 6). They showed a very slight response to sucrose, salt and acid, and no response to quinine.

Type VII

Only three single functional fibres, which responded to no other test solutions than to weak acetic acid were found.

Tap water sometimes produced a response of fibres which were spontaneously active and which were silenced by the application of saliva, and such a spontaneously active fibre was silenced by the application of 0.5 M NaCl, while its activity was enhanced by the application of 0.5 M sucrose. In addition, water sometimes also produced a moderately strong response when applied shortly after a previous application of acid as well as saliva. Repeated application of water, however, had no effect on these fibres, in contrast to the effect of repeated applications of water on the tongue of the frog or the cat.

Thresholds determined on the entire glossopharyngeal nerve for the different taste substances were low. The salt threshold was less than 0.0005 M, that of sugar ranged from 0.01 M to 0.005 M (rarely 0.001 M) and that of quinine ranged from 0.0005 M to 0.0002 M.

Discussion

The palatal organ as well as the gill rakers display a differentiated chemical sensitivity as compared to that of the skin in general. This is in good accordance

with the dense distribution of taste buds in this organ and our finding of fibres responding to the application of sucrose and quinine solutions, which do not stimulate the endings of the common cutaneous nerve fibres.

A comparison of the chemoceptive response of the palatal nerves with that from the nerves innervating the lips, the barbels and the gill rakers gives us the impression that the palatal organ is the most important gustatory organ for feeding in this fish. It is quite interesting that although amphibian frog has "water" fibres, aquatic fish apparently does not. The fact that water in which fishes had been kept stimulates the chemoceptors while pure water does not implies a highly developed gustatory sensitivity in fish. Although we could not as yet identify the stimulants dissolved in this water, mucin and skin fluids possibly may serve as the effective agents. In this connection it is interesting to note the behavioural observations demonstrating that fishes are able to detect the presence of another species or of wounded fish.

The integrated responses to salts were usually rather smaller in magnitude than those to sugar and even to saliva. We cannot, however, argue about the quantitative comparison of the integrated responses, because the magnitude of the response is influenced not only by the number of active fibres and their impulse frequencies but also by the size and configuration of the individual spikes.

The existence of many fibres displaying a broad gustatory spectrum, as was described above, directs the attention to the possibility of a simple osmotic effect as the primary mechanism in producing taste sensation in the fish. This possibility, however, is ruled out because sugars are more effective than many electrolytes.

Among the taste substances which stimulate the palatal organ, the most powerful is acid and next to that sugar. Specific "acid" fibres seemed to be few in total number, but acid stimulates the majority of the gustatory fibres.

The effect of human saliva is rather interesting. In spite of being tasteless to humans, saliva vigorously stimulated many kinds of fibres in the fish, including sweet fibres which were sometimes stimulated even stronger than by sugar. To our surprise, we even found a few fibres specifically responding to saliva. Saliva, of course, contains many substances, such as proteins (including an enzyme and mucin), salts etc. But saliva does not stimulate salt fibres. It is rather difficult to identify the effective substances because of the complicated nature of saliva, and this problem will be dealt with in a subsequent paper. The extract of mucous substances covering the body surface of the fish also stimulated the taste nerves, as already described. It is notable that the sweet fibres were found to be stimulated by the mucous substances as well as by saliva.

It is obvious that the great majority of taste fibres running to the palatal organ of the carp display a rather broad gustatory spectrum. This raises again the question previously brought up by PFAFFMANN (1941), as well as by one of us, whether a taste fibre might possess in its endings different receptors re-

sponding to different classes of gustatory stimuli. As it is difficult to find any common denominator for the excitatory effect of such stimuli as sugar, salt, quinine and acid, it seems more likely that the broad gustatory sensitivity of these fibres is due to the existence of different chemosensitive sites in the ending fibrilles of the individual gustatory nerve fibre. Such an arrangement would thus be similar to Granit's system of dominators in the retina. Strictly specific taste fibres, corresponding to Granit's modulators, we have found only to salt (Type IV a, 14 fibres) and to acid (Type VII, only 3 fibres) and to human saliva (Type VI, only 3 fibres). In addition to these we have the Type V fibres (13 fibres), which respond only to quinine and acid. From these findings it is possible to picture a mechanism enabling the fish to elaborate a fairly high level of gustatory discrimination. Behavioural studies on fresh water fishes have also revealed that these fishes discriminate between the four basic classes of tastes (Uchida 1933). Further, the high sensitivity of the palatal organ to human saliva, as well as to the mucous covering the scales of fish, would suggest that the taste of these fishes may serve its specific purposes, in addition to their olfactory mechanism, in the search for food and in detecting other individuals.

Our finding of a relatively great number of taste fibres responding to sweet tasting substances is in close agreement with behavioural experiment on fresh water fishes. Trudel (1929) made an extensive study of the taste reactions of minnows (*Phoxinus laevis*). He determined the threshold concentration for saccharose to 0.01—0.005 M, while Uchida (1933) gave a value as low as 0.0002 M. The threshold of 0.01—0.005 M which we found for the electrical response is thus in good agreement with Trudel's figures. The threshold for sucrose in man is generally given as 0.01 M which implies that the carp's sensitivity for "sweet" may be equally strong as that of humans. The lower threshold found by Uchida may depend on individual differences as well as on the age of the fish. We have every reason to suppose that there may exist as much of species and individual differences in fish as there are between mammals and it is well known that a pike does not snatch at a bait of sweet dough as does a minnow or a carp.

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The Role of the Adrenals on the Mucosal Mast Cells and Tissue Eosinophils in the Gastric Wall of Rat

By

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Abstract

RÄSÄNEN, T. *The role of the adrenals on the mucosal mast cells and tissue eosinophils in the gastric wall of rat.* Acta physiol. scand. 1961. *52.* 162—166. — The effect of ACTH on the mucosal mast cells and tissue eosinophils in the gastric wall of adrenalectomized and intact rats was studied. In intact rats, ACTH caused almost complete degranulation of the mucosal mast cells and a highly significant fall in tissue eosinophilia. Its degranulating effect on the mucosal mast cells and its tissue eosinopenic effect were inhibited after adrenalectomy. Adrenalectomy alone caused a slight increase in both these cell types in the gastric mucosa. The significance of mast cells as a mucosal histamine depot and its possible primary role as secretory stimulant of the parenchyma and secondary role in causing reduction of tissue eosinophilia is discussed.

The degranulation of the mucosal mast cells in the gastric mucosa of rat is a rapid and quantitative process under the influence of glucocorticoids. Mineralocorticoids do not seem to have a degranulating effect on these cells (RÄSÄNEN 1961). The location of mucosal mast cells in the superficial part of the gastric mucosa where the histamine content is greatest (FELDBERG and HARRIS 1953) suggests that histamine conjugated with the polysaccharides of the mast cell granules constitutes the histamine depot of the gastric mucosa.

The degree of tissue eosinophilia in the lamina propria of the stomach may increase (WEGELIUS and TEIR 1958) or decrease (RÄSÄNEN 1960 a) after a glucocorticoid injection, depending presumably on the rate of eosinophil consumption in the reactions stimulated in the lamina propria and on the compensating capacity of myeloid and possible local eosinopoiesis. Histamine released from the tissues under the influence of glucocorticoids (SCHAYER et al. 1954) causes a drop in blood eosinophiles immediately (DUNÉR and PERNOW

Table I. Number of mucosal mast cells and tissue eosinophils ± standard error per sq.mm in the gastric wall of intact and adrenalectomized rats with and without ACTH-therapy

	Adrenalectomized		Intact	
	ACTH	Saline	ACTH	Saline
Mast cells	911 ± 101	1,345 ± 122	4.8 ± 1.9	945 ± 121
Eosinophils	2,824 ± 99	3,564 ± 301	247 ± 65	2,550 ± 158

1958, Rose et al. 1958) or by occasioning the release of endogenous histamine through stimulation of the pituitary-adrenal system (Halpern and Bénos 1951, Lecomte et al. 1959, Zaratzian 1959).

The conjugation of glucocorticoids takes place to a great extent in the gastrointestinal canal — the reaction is inhibited in deintestinalised rats and is retarded when the portal circulation slows down in hepatic cirrhosis. It has been established that in the latter case the eosinopenic response to Thorn's test is also reduced (Wahi and Ramachandran 1958).

ACTH has been established earlier to have a degranulating effect on mucosal mast cells and a reducing influence on tissue eosinophilia (Räsänen 1960 a, b). This effect probably occurs through stimulation of the adrenal cortex. The mucosal mast cell count must perhaps be expected to rise after adrenalectomy when corticoid secretion is absent and the tissue eosinopenic effect is inhibited.

Method

Male rats, age 4 months and weight 146—220 g, of Dawley-Sprague strain were used in the investigation. Adrenalectomy was performed on 20 rats after the animals were adapted to the conditions in the laboratory. Ten adrenalectomised and 10 intact rats were given 4 IU of ACTH (Cortrophine-Z, Organon) intramuscularly once a day for 10 days. Ten adrenalectomised and 11 intact rats were given 0.5 ml saline injections daily for the same period. Food and water were given ad libitum; the adrenalectomized animals were given saline to drink. Decapitation was performed 24 hours after the last injection during which time the rats fasted and were kept in cages with a net floor.

The rat stomachs were removed immediately after decapitation and split longitudinally in two. The halves were fixed in formalin and in basic lead acetate. The sections, staining and cell count were performed as reported earlier in detail (Räsänen 1960 a, b).

Results

The cell count is given in cells per square millimetre of tissue. The values are shown in Table I and Fig. 1.

Metachromatically granulated mucosal mast cells disappeared almost completely in intact rats given ACTH ($P < 0.001$), probably owing to their degranulation. No degenerative changes were demonstrable in the nuclei of even extensively degranulated mucosal mast cells. A corresponding degranulation under the influence of ACTH was not established in the submucosal mast cells.

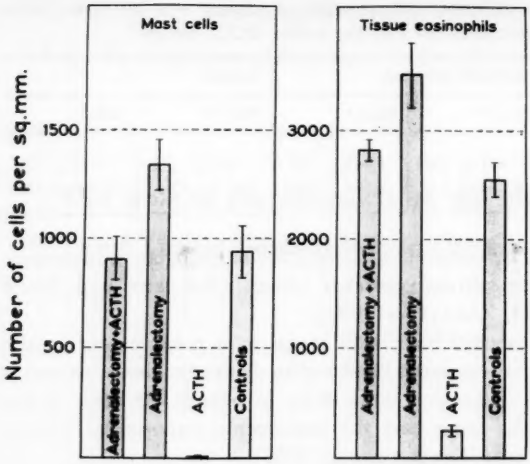

Fig. 1. Diagram of the amount of mucosal mast cells and tissue eosinophils in rat stomach wall after adrenalectomy and ACTH application.

The degranulating effect of ACTH on mucosal mast cells seemed to be inhibited after adrenalectomy ($P < 0.02$). Although it was possible in this group to count mast cells in the stomach of two rats only in which no superficial mucosal lesions occurred, their mucosal mast cells did not reveal even partial degranulation or vacuolisation, both of which were so profuse in the mucosal mast cells of the intact rats treated with ACTH. The amount of mucosal mast cells increased ($P < 0.05$) after adrenalectomy and their granulation was intensive.

Tissue eosinophilia diminished very distinctly ($P < 0.001$) during ACTH therapy in the basal part of the lamina propria. This phenomenon disappeared after adrenalectomy and even changed to a slight increase ($P < 0.05$) in adrenalectomized rats.

Discussion

The increase in the amount of mucosal mast cells and the intensification of their granulation probably indicate that the consumption of polysaccharides, containing glucuronic acid, under the influence of endogenous glucocorticoids in the intact animals is inhibited after adrenalectomy. The mucosal stores of mast granules which are augmented in this connection are perhaps able to bind greater quantities of histamine. The amount of histamine increases after adrenalectomy, especially in the gastrointestinal canal (BARTLET and LOCKETT 1959).

ACTH degranulated the mucosal mast cells fairly thoroughly during the experimental period of 10 days. This process obviously took place through the mediation of adrenocortical stimulation. It was attributed to the mobilisation of glucocorticoids since no comparable phenomenon was produced by mineralocorticoids (RÄSÄNEN 1961).

Glucocorticoids stimulate gastric secretion (GRAY et al. 1951, PLAINOS & PHILIPPU 1958, PEREMANS 1958), and also histamine liberators (PATON and SCHACHTER 1951, PICCALUGA et al. 1958). The stimulating effect of histamine on gastric secretion is inhibited in Addison's disease (THORN et al. 1951). It is possible that the stimulation of the mucosal parenchyma takes place through the release of the histamine of the mucosal mast cells. This stimulation is probably so immediate in the mucosa that antihistamines are incapable of inhibiting it (HALPERN and MARTIN 1946); for instance, the stimulation occurring by this route is increased by aminoguanidine (IVY and LIEPINS 1960), a histaminase inhibitor. Gastric secretion is inhibited by hibernation (GILLESPIE 1956). The degranulation of the mast cells (HÖGBERG and UVNÄS 1957, UVNÄS et al. 1960) and the histamine release (SZILÁGYI et al. 1960) are also inhibited in the same connection.

ACTH caused a fairly complete loss of tissue eosinophilia. This loss obviously occurs also through the stimulation of the adrenal cortex. The drop in blood eosinophilia is dependent on the amount of ACTH (SPEIRS et al. 1959), and tissue eosinophilia of rat stomach decreases in correlation with the amount of glucocorticoids (RÄSÄNEN 1961). Tissue eosinopenia emerges, however, more slowly than the corresponding degranulation of the mucosal mast cells. Prolonged ACTH therapy causes in man retention of eosinophils in the blood and inhibition of response to Thorn's test (MINOZZI 1959), probably because of the diminution of the polysaccharide-histamine depot of the tissues.

Blood eosinophilia is lowered in intact and adrenalectomized rats by injecting a foreign serum, gammaglobulins and many aminoacids (ASCHKENASY 1959) which are histamine liberators (PATON 1957) into the great mast cell depot of the abdominal cavity. The same chain of events probably occurs in the gastric mucosa under the influence of ACTH-stimulated glucocorticoids: conjugation of glucocorticoids — release of histamine — eosinopenia. Stimulation of the parenchyma is associated with this chain of events.

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Uptake of Dopamine by the Storage Granules of the Adrenal Medulla in Vitro

By

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Abstract

BERTLER, Å., G. HALL, N.-Å. HILLARP and E. ROSENGREN. *Uptake of dopamine by the storage granuels of the adrenal medulla in vitro.* Acta physiol. scand. 1961. *52.* 167—170. — Large amounts of dopamine are taken up by the amine storage granules of the cow adrenal medulla when the granules are incubated at + 31° in a solution containing dopamine. At this temperature the amine accumulates even against an apparent concentration gradient but at 0° only small amounts — if any at all — are incorporated. The amine seems to be bound in the granules but is released on osmotic lysis of the granules.

Dopamine (DA) is rapidly taken up the amine storage granules of the adrenal medulla when formed *in vivo* in the cells (BERTLER, HILLARP and ROSENGREN 1960). It has now been found that these granules are capable of incorporating DA also *in vitro*.

Material and Methods

Amine granules from cow adrenal medulla were isolated in 0.3 M sucrose as described previously (HILLARP 1958 a). They were not washed and were thus contaminated with other cell particles (*i. a.* mitochondria) and, to some extent, also with cytoplasmic sap. Immediately after their isolation the granules (100—150 mg wet weight/ml) were suspended in a solution of DA — HCl (10—20 μmoles/ml) containing (in μmoles/ml) ascorbic acid 20, potassium chloride 40, magnesium chloride 5, sodium phosphate (pH 7.5) 15 and sucrose 180. The pH was adjusted to 7—7.5 with potassium carbonate immediately before the use of the solution. Incubation was performed without shaking at + 31° for one hour, after which the suspension was chilled to 0°, diluted 40 times with

Table I. Total amounts of catecholamines in the supernatant (Sup.) and in the granule sediment (Gran.) after incubation of amine granules in a medium containing DA.
After the incubation the granule suspensions were either directly centrifuged or first diluted 40 times with 0.5 M sucrose (or 0.1 M sucrose for osmotic lysis) and then centrifuged

Incubation temperature	Treatment after the incubation	Cell fraction	A µg	NA µg	Changes in A + NA µg	DA µg	A + NA + DA µg
0°	No dilution	Sup.	175	100		1,370	
		Gran.	2,050	1,500		165	3,700
0°	Diluted 40 ×	Gran.	2,050	1,400		70	3,500
+ 31°	No dilution	Sup.	605	280	+ 610	850	
		Gran.	1,750	1,220	− 580	595	3,550
+ 31°	Diluted 40 ×	Gran.	1,650	1,150	− 650	460	3,250
+ 31°	Osmotic lysis	Gran.	13	70		20	

cold 0.5 M sucrose and centrifuged at 38,000 × *g* for 30 min. The catecholamine content of the granule sediment and of the supernatant was determined spectrophotofluorimetrically (BERTLER, CARLSSON and ROSENGREN 1958, CARLSSON and WALDECK 1958). To obtain pure amine granules, density gradient centrifugation of granules incubated as described above was made by a method previously described (HILLARP 1958 a).

Results and Discussion

Large amounts of DA — up to about 20 per cent of the amounts of adrenaline (A) + noradrenaline (NA) in the granuels — were regularly recovered in the granule sediment after an incubation at + 31° for one hour. The data from a typical experiment are found in Table I. That this "bound" DA had in fact been taken up by the amine storage granules was shown by means of density gradient centrifugation. The DA showed the same distribution as that of the storage granules. About 50 per cent of the DA as well as of the A + NA was recovered in the sediment containing pure amine granules with the highest density, in which the amines are normally stored together with an equivalent amount of adenosinephosphates (cf. HILLARP 1960 a).

The experiments indicate (see Table I) that DA — just as A and NA — can penetrate the granule membrane from without at 0° and reach about the same concentration in the intragranular water as in the medium. At this temperature, however, only small amounts — if any at all — of DA were incorporated, in agreement with previous findings (CARLSSON and HILLARP 1958, HILLARP 1959). The fact that the storage process is inhibited at lower temperatures does not necessarily mean that it is an active, energy-utilizing process. It is in

fact equally probable that this phenomenon is caused by a change in the state of the storage complex (cf. HILLARP 1960 b). At 0° the complex may well be more "condensed", and thus prevent amines from penetrating it.

The experiments clearly show that at + 31° the granules are capable of accumulating DA even against an apparent concentration gradient. The incorporated DA is "bound" in such a way that it is not released but held for at least 24 hours if the granules are kept suspended in 0.5 M sucrose at 0° in spite of a very high difference of DA concentration (more than a hundredfold) between the granules and the medium. Since it does not seem possible that an active transport mechanism is responsible for the maintenance of the steep concentration gradient, it seems reasonable to assume that the amine is kept in a bound state, perhaps in a way similar to that of the stored A and NA. There is another striking similarity between the ways in which the three amines are stored, since the DA — just as the A and NA — is immediately released on osmotic lysis of the granules (Table I).

Experiments with incubation media of different compositions have not supported the view that ascorbic acid, magnesium chloride etc, which usually were included, are essential for the amine incorporation. Adenosinediphosphate (15 μ moles/ml) had no obvious effect. In fact, DA was readily taken up from a solution containing only DA—HCl in 0.3 M sucrose (neutralized with potassium carbonate).

Even when large amounts of DA were incorporated in the granules, no increase in the total amount of stored amines was observed (Table I). On the contrary, a loss usually occurred. This is easily understood on the basis of the fact that the stored A and NA are released when the granules are kept at elevated temperatures (HILLARP 1958 b). In the present work it was found that this "spontaneous" release is not materially influenced by the presence of DA in the suspension medium. Thus it is not possible at present to decide whether the storage of DA is a result of an exchange between this amine and the stored A and NA, and not even whether the two processes: storage of DA and release of A and NA, are coupled together.

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The Fate of Uniformly Labelled ^{14}C Fructose in Different Parts of the Brain and the Pituitary in Goats

By

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Abstract

LARSSON, S. *The fate of uniformly labelled ^{14}C fructose in different parts of the brain and the pituitary in goats.* Acta physiol. scand. 1961. *52.* 171—177. — The fate of uniformly labelled ^{14}C fructose has been studied in different parts of the brain and in the anterior and posterior pituitary. Generally, the metabolism of fructose was less than that of glucose. Particularly in the anterior part of the pituitary the fructose utilisation was small. As the oxygen consumption was little affected by the fructose, the utilisation of endogenous substances must be increased compared to experiments where glucose was used as substrate. The rate of conversion of fructose into various amino acids was considerably lower in the periventricular parts of the hypothalamus and in the anterior part of the pituitary as compared to the glucose experiments. The observed differences are discussed in relation to the importance of the pentose shunt.

BELOFF-CHAIN *et al.* (1955) investigated the fate of ^{14}C-labelled fructose in slices of rat brain by means of quantitative radio-chromatography. No differences were found except for a generally lower metabolism as compared to glucose. Further, previous studies have shown that marked differences exist in the fate of ^{14}C-labelled glucose in various parts of the brain and in the anterior and posterior parts of the pituitary (CHAIN, LARSSON and POCCHIARI 1960, ANDERSSON, LARSSON and POCCHIARI 1961).

The present study was undertaken to see if the various parts of the brain and the pituitary also showed the above mentioned differences when fructose was used instead of glucose.

Table I. Oxygen uptake, CO_2 and lactic acid formation in different parts of the brain and the pituitary after incubation with ^{14}C fructose or ^{14}C glucose

Results expressed as μl of oxygen uptake per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. CO_2 and lactic acid expressed as μg fructose or glucose converted, based on the fraction of total radioactivity of fructose or glucose incorporated under the conditions mentioned above. Fructose or glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values ± s. e. m. Values within brackets derived from ANDERSSON et al. (1961).
Differences statistically significant $p < 0.05$

Tissue		O_2	CO_2	Lactic acid	No. of exper.
Periventr. area	Fr.	35.3 ± 2.6	4.5 ± 0.5	31.1 ± 2.0	5
	Gl.	(38.7 ± 3.1)	[1](11.9 ± 1.0)	[1](102.3 ± 7.2)	14
Ventromed. area	Fr.	32.2 ± 4.0	4.9 ± 1.4	38.6 ± 2.9	5
	Gl.	(34.0 ± 3.3)	[1](10.8 ± 1.9)	[1](89.8 ± 4.3)	10
Cerebral cortex	Fr.	33.0 ± 4.1	9.2 ± 1.6	23.4 ± 2.2	5
	Gl.	36.2 ± 3.7	10.5 ± 1.4	[1]103.3 ± 9.7	6
Posterior pituitary	Fr.	26.2 ± 3.3	5.2 ± 1.3	33.3 ± 2.4	5
	Gl.	30.3 ± 3.2	(8.7 ± 1.6)	[1](72.3 ± 5.1)	10
Anterior pituitary	Fr.	20.1 ± 1.7	0.4 ± 0.1	5.5 ± 1.8	6
	Gl.	(26.1 ± 2.6)	[1](2.3 ± 0.3)	[1](30.3 ± 1.3)	8 & 9

[1] = differences statistically significant
Gl. = glucose, Fr. = fructose

Methods

Generally labelled ^{14}C fructose and ^{14}C glucose were obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give a specific activity of 20 μC per mg.

The incubation medium was the same as that used by ANDERSSON *et al.* (1961) a phosphate buffer with the radioactive material 0.0056 M, containing 3.6 mC/mmole at pH 7.3. Adult female goats ded *ad libitum* were used in the experiments. The animals were killed by decapitation and the samples dissected out as quickly as possible.

The following samples from the brain and the pituitary were taken:

1. Periventricular tissue
2. Ventromedial tissue
3. Parts of the posterior pituitary
4. Parts of the anterior pituitary
5. Parts of the cerebral cortex, taken medially just caudal to the frontal lobes.

Parts 1—4 included the same areas as described earlier (ANDERSSON *et al.* (1961)).

After incubation of the tissue for one hour at 37° C — the gas phase being pure oxygen — the samples were treated in exactly the same way as previously described (CHAIN *et al.* 1960, ANDERSSON *et al.* 1961). As in the previous experiments the oxygen consumption was measured every 15 min. After incubation the former Na_2CO_3 was collected and precipitated with $BaCl_2$ as described by VILLEE and HASTINGS (1949).

Table II. Amino acid formation from metabolised ^{14}C fructose or ^{14}C glucose in different parts of the brain and the pituitary

Results expressed as μg fructose or glucose converted per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. Fructose or glucose concentration 0.1 %; total radioactivity 10 μg per vessel. Mean values ± s. e. m. Values within brackets derived from ANDERSSON et al. (1961).
Differences statistically significant $p < 0.05$

Tissue		Alanine	Aspartic acid	Glutamic acid	GABA	Glutamine	Arginine
Periventr. area	Fr.	0.9±0.1	0.5±0.1	2.9±0.4	2.2±0.3	0.6	0.3±0.1
	Gl.	(0.6±0.1)	(0.9±0.1)	[1](6.0±0.7)	(3.5±0.3)	(1.2±0.3)	(0.9±0.2)
Ventromed. area	Fr.	1.0±0.02	0.7±0.2	3.2±0.4	2.3±0.3	0.6	trace
	Gl.	(1.3±0.3)	(0.5±0.1)	(4.4±0.6)	(2.6±0.5)	(1.1±0.2)	(0.4±0.1)
Cerebral Cortex	Fr.	0.6±0.1	1.1±0.4	4.0±0.8	1.0±0.3	0.4	trace
	Gl.	1.4±0.5	0.8±0.2	6.4±0.8	1.3±0.3	1.5±0.4	trace
Posterior pituitary	Fr.	0.6±0.1	0.5±0.1	4.4±0.6	0.8±0.2	0.3	0.3±0.1
	Gl.	(0.7±0.2)	(0.8±0.1)	(4.1±0.5)	(1.0±0.03)	(0.7±0.1)	(0.7±0.2)
Anterior pituitary	Fr.	0.1	trace	0.1	—	0.2	0.2
	Gl.	[1](0.9±0.1)	[1](0.4±0.05)	[1](0.7±0.1)	—	[1](0.9±0.2)	(0.4±0.05)

[1] = differences statistically significant
Gl. = glucose, Fr. = fructose; GABA = γ-aminobutyric acid
Number of experiments the same as in Table I.

The radioactive metabolites formed from the glucose and fructose were separated by paper-chromatography as described earlier (CHAIN *et al.* 1960). All chromatograms were scanned quantitatively by a modification of the automatic scanning device according to FRANK *et al.* (1959).

Results

Table I shows the oxygen consumption and the formation of CO_2 and lactic acid in different parts of the brain and the pituitary when the samples were incubated with ^{14}C fructose or ^{14}C glucose. The "fructose"-values were generally lower than the "glucose" ones. Except for the values on the oxygen consumption the differences were statistically significant. When the tissue was incubated with fructose the oxygen consumption was lower than when incubated with glucose. In the hypothalamic samples and the tissue from the posterior pituitary the decrease in CO_2 and lactic acid production was essentially the same when fructose was present in the substrate. Thus, the CO_2 formation for these three parts was only half, and the lactic acid formation about one third of the values found for glucose. In the experiments on the anterior pituitary the corresponding differences were considerably larger. In the cerebral cortex the decrease when using fructose was most pronounced in the lactic acid production.

Table III. The occurence of free unchanged ^{14}C fructose or ^{14}C glucose in the cells of different parts of the brain and the pituitary

Results expressed as μg fructose or glucose per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. Fructose or glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values $\pm$ s. e. m.

Tissue	Incubation medium		
	^{14}C fructose		^{14}C glucose
	Free fructose	Glucose formed from fructose	Free glucose
Periventricular area	8.3 $\pm$ 1.2	0.2	8.3 $\pm$ 1.3
Ventromedial area	7.3 $\pm$ 0.8	0.3	3.3 $\pm$ 0.4
Cerebral cortex	6.7 $\pm$ 0.8	0.4	5.8 $\pm$ 0.7
Posterior pituitary	15.3 $\pm$ 2.5	0.9	8.3 $\pm$ 1.4
Anterior pituitary	11.5 $\pm$ 1.5	0.3	12.0 $\pm$ 2.3

Table II shows the amounts of ^{14}C fructose and ^{14}C glucose converted into the various amino acids, found after extraction of the samples from the different parts of the brain and the pituitary. Generally there was a decreased total amino acid formation from the fructose in the medium as compared to glucose. This was particularly evident in the samples from the anterior pituitary and from the periventricular tissue. In these parts the amino acid formation from the fructose in the medium was so small that in the anterior pituitary not more than traces could be found. There was a tendency for lowered conversion of fructose into glutamic acid and γ-aminobutyric acid in all samples studied (except for glutamic acid in the posterior pituitary). The formation of alanine and aspartic acid did not seem to be changed when fructose was present in the medium compared to glucose. Due to analytical difficulties in separating ^{14}C fructose and ^{14}C glutamine chromatographically the values of the amount of fructose converted into glutamine were not as reliable as desired. It seemed, however, that the amount of glutamine found in this case was less than the amount formed from glucose. Table III shows the tendency for a greater accumulation of the fructose in the cells as compared to the glucose experiments. The same table also shows that ^{14}C glucose can be formed and found in the cells after incubation of the samples with ^{14}C fructose. In addition some glucose was also found in the incubation medium in these experiments. Generally the amount of unchanged fructose found in the cells after incubation was about 20 % of the total amount of fructose disappearing from the medium.

The radioactivity found in the insoluble residues after incubation with fructose were not significantly different from those formed with glucose.

Discussion

McIlwain (1953 a, b), studying the possible role of fructose as a substrate for the brain, found that the respiratory rate of cerebral cortex with equimolar concentrations of glucose or fructose was higher with glucose than with fructose, provided the concentrations of the two substrates were equivalent to 5 mM or less. In the present experiments where parts other than the cerebral cortex were also studied similar observations were made. While the respiratory exchange of the cerebral cortex was only little affected by fructose in the present experiments, the differences between fructose and glucose were more pronounced in the other parts studied with the exception of the posterior pituitary. This might imply that the hypothalamus and the anterior pituitary in particular are more dependent on the endogenous supply of oxidisable material when fructose is the substrate than when glucose is present in the incubation medium. In this connection, however, it is of interest to note that the $^{14}CO_2$ formation of the anterior pituitary in relation to oxygen consumption is also very low when glucose instead of fructose is present in the substrate (Table I and Andersson *et al.* 1961). From the values obtained from the glucose experiments it is further evident that *in vitro* more than 50 % of the CO_2 formed is derived from endogenous oxidation. However, fructose will generally increase the endogenous oxidation in the tissue from the hypothalamus and the pituitary (Table I). Even if the total uptake of fructose is less than that of glucose it is evident when regarding the values for the CO_2 and lactic acid formation that both are useful substrates for the different parts of the brain and the posterior pituitary.

Beloff-Chain *et al.* (1955) incubated brain slices with ^{14}C-labelled fructose and found the same general metabolic pattern as when ^{14}C glucose was used. However, they found the overall metabolism was lower for fructose than for glucose. Further, it has been demonstrated in intact animals that there is a limited penetration of fructose into cerebral tissue (Stone 1938, Klein and Olsen 1947). After parenteral administration of ^{14}C fructose the uptake in different parts of the brain was lower than for glucose (Larsson and Lepkovsky, *in press*). The uptake of labelled fructose as well as of glucose could, however, be increased by the deprivation of food. In the present experiments the conversion of labelled fructose into CO_2 and lactic acid was considerably lower than when glucose was used. Concerning the CO_2 this was particularly pronounced in the samples from the hypothalamus and from the anterior pituitary. As mentioned before the values for oxygen consumption and the $^{14}CO_2$ production after incubation with labelled fructose indicated a relative increase in the utilisation of endogenous oxidisable substances. Further, in comparison with the present results demonstrating a lower ^{14}C lactic acid formation with fructose it has been found that in cerebral tissue fructose and

mannose can yield lactate as rapidly as can glucose but that higher concentrations are required (GEIGER 1940, MEYERHOF and WILSON 1948).

BELOFF-CHAIN *et al.* (1955) found that approximately 18 % of the total fructose uptake by brain slices was accounted for by free unchanged fructose in the cells. In the present experiments the same observation was made. The corresponding figure for the cerebral cortex being about 14 % but in the anterior pituitary the amount of free unchanged fructose was considerably higher. When calculating the absolute amounts of free unchanged fructose in the cells of the different parts studied there was no essential differences within the brain or between the two hypophyseal samples. It therefore seems reasonable to assume that it is not the transport mechanism for fructose suggested by BATTAGLIA and RANDLE (1959) which is responsible in this case for the differences observed in the fate of fructose and glucose in the various samples studied. In accordance with the findings by BELOFF-CHAIN *et al.* (1959) small amounts of ^{14}C glucose were formed from the labelled fructose in the medium. The explanation for the differences in fructose utilisation in the different samples studied is not fully established. It should be pointed out, however, that evidence has been presented for the existence of a hexose monophosphate pathway for glucose oxidation in endocrine organs (FIELD *et al.* 1960). Further, with the same experimental technique used in the present study, these findings are supported (HELLMAN and LARSSSON, to be published). It also seems as certain parts of the hypothalamus should have a more active pentose shunt.

In brain slices, BELOFF-CHAIN *et al.* (1955) found that fructose qualitatively followed the same metabolic pattern as glucose. Thus, besides a generally lowered conversion of fructose into the different amino acids, no distribution differences were observed when compared to glucose. In the present experiments this observation was generally confirmed. Certain distribution differences existed however, with regard to the amino acid formation. Thus, in the periventricular tissue and in the anterior pituitary the conversion of fructose into glutamic acid was proceeding at a comparatively lower rate when compared to the other amino acids found. As mentioned previously the amino acid formation in the anterior pituitary from the medium fructose was very low also for the other amino acids. As was the case for the CO_2 formation in this part a corresponding increase in the "endogenous" formation of the amino acids could take place. Except for the anterior pituitary, the decreased conversion of fructose into amino acids was less pronounced compared to the very marked decrease in CO_2 and lactic acid formation in most samples studied. It is therefore possible that the amino acid pool is very little affected by fructose as the substrate compared to glucose.

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Acta physiol. scand. 1961. 52. 178—184

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The Influence of Rapid Injection of Concentrated Potassium Chloride Solution on the Cardiovascular Functions of the Cat in Respiratory Disorders

By

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Abstract

ANTILA, L. and K. HARTIALA. *The influence of rapid injection of concentrated potassium chloride solution on the cardiovascular functions of the cat in respiratory disorders.* Acta physiol. scand. 1961. *52.* 178—184. — The effect of concentrated potassium chloride solution on the cardiovascular functions of the cat has been studied under severe asphyctic, hypoxic, and hypercapnic conditions. The potassium concentrations varied between 330—670 meq/l and the total amount injected between 0.33—0.67 meqK^+ or 0.15—0.35 meqK^+/kg b. w. The blood oxygen saturation and pH as well as serum potassium content were analysed before the injection. The effect of the injection was followed by continuous electrocardiogram and aortic pressure recordings. The 30 min period of respiratory disturbance did not cause any uniform changes in the serum potassium content. The cardiovascular changes prior to the injection were also unremarkable. The results indicate that the tolerance of the cat towards high potassium concentrations is mainly depressed by lack of oxygen whereas an excess in carbon dioxide does not appear to be so dangerous.

Respiratory disturbances have shown to cause at least in dogs changes in the distribution of potassium. It has been found that potassium is mobilized from the cells during tissue anoxia (DENNIS and MOORE 1938, MULLIN *et al.* 1938) in respiratory acidosis (SCRIBNER *et al.* 1954, BURNELL *et al.* 1956) and

during respiratory alkalosis (GIEBISH *et al.* 1955, HICKAM *et al.* 1956). MULLIN *et al.* (1938) have claimed the serum potassium increase rather being due to the oxygen lack than the carbon dioxide excess. An increase in the potassium has been shown to take place in pure respiratory acidosis (SCRIBNER *et al.* 1954). In the present investigation studies have been made of the tolerance of the cat towards acute concentrated potassium injections during various respiratory disturbances.

Material and Methods

Cats of both sexes weighing 1.5—4.4 kg were used in the experiments. The material was divided into two groups. In the first one the potassium tolerance was studied in the same cats subjected to different disturbances. In the other group each cat received the same potassium injection under a certain disturbance condition.

The animals were anesthetized with 0.07 g/kg of nembutal given intraperitoneally. The tracheal cannula was connected into a 250 l plastic bag containing different gas mixtures. These were in the asphyxia experiments 7 % O_2, 13 % CO_2 and 80 % N, in the anoxia experiments 7—10 % O_2, 0—3 % CO_2 and 7—90 % N and in the hypercapnia experiments 20—80 % O_2, 13—20 % CO_2 and 0—67 % N. The expiration was conducted by means of valves into the room air. It was also possible to allow the cat breath through the valves regular room air.

The II lead was used in the ECG registration. The pressure recordings were made from the femoral artery through a catheter, which was brought up to the region of the thoracic aorta. Blood samples were also taken through this catheter. Another catheter was introduced through the femoral vein 15 cm into the inferior caval vein. The potassium was administered through this catheter.

From the blood samples the hemoglobin, oxygen saturation, potassium concentration and the arterial pH and also in some case the blood oxygen and carbon dioxide contents were determined.

Results

General. Preliminary experiments were performed on 3 cats which were exposed to asphyxia and hypoxia over a period of 60 min by using different gas mixtures. By reducing the oxygen content in these mixtures to 7 % a marked drop down to 75—52 per cent was achieved in the oxygen saturation. The results were similar after 30 and 60 min with regard to the cardiovascular changes and laboratory analyses and the cats tolerated well the condition. Since it has been shown in dogs that oxygen is harmful in respiratory acidosis (MILLER *et al.* 1952, SCRIBNER *et al.* 1954) pure oxygen was given to these cats after the exposure to asphyxia. No harmful side-effects could be noted.

Laboratory analyses. The low oxygen saturation values in hypoxia and asphyxia in Fig. 1 indicate that the condition has really been grave. No consistent changes in the serum potassium concentrations were found since in some cases there was a rise whereas in some cases even a drop was present and there were values below the control values particularly in connection with hypoxia. In some cases potassium had been administered before the

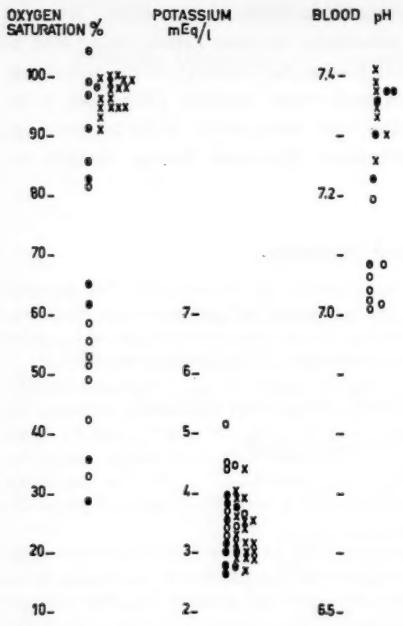

Fig. 1. Laboratory data on cats in atmosphere and in different respiratory disorders.
× atmosphere, ○ asphyxia,
● hypoxia, ⊙ hypercapnia.

appearance of serum potassium concentrations higher than control values. The blood pH analyses showed that the animals were in deep acidosis both during asphyxia and hypercapnia. This acidosis has been of the respiratory type as indicated by the occasional blood carbon dioxide determinations and as such what could be expected under these experimental conditions. In few cases the low pH values at the beginning of the experiment under normal atmospheric conditions may be interpreted as a result of hypoventilation caused by the anesthesia.

The general cardiovasculatory observations in various respiratory disturbances showed only minor changes. The most consistent change was the rise in the pulse pressure. It had dropped only in two cases and remained unchanged in another two. The systolic pressure frequently increased, and in all cases in hypercapnia.

The changes in the ECG were of no significance. Such were more frequent in the first experimental series, obviously because in these potassium was administered between the registrations whereas in the latter series the animals were only exposed for respiratory disturbances before the ECG control.

The effect of potassium injections on the cardiovasculatory functions. Potassium, when administered rapidly and in concentrated solutions, caused similar

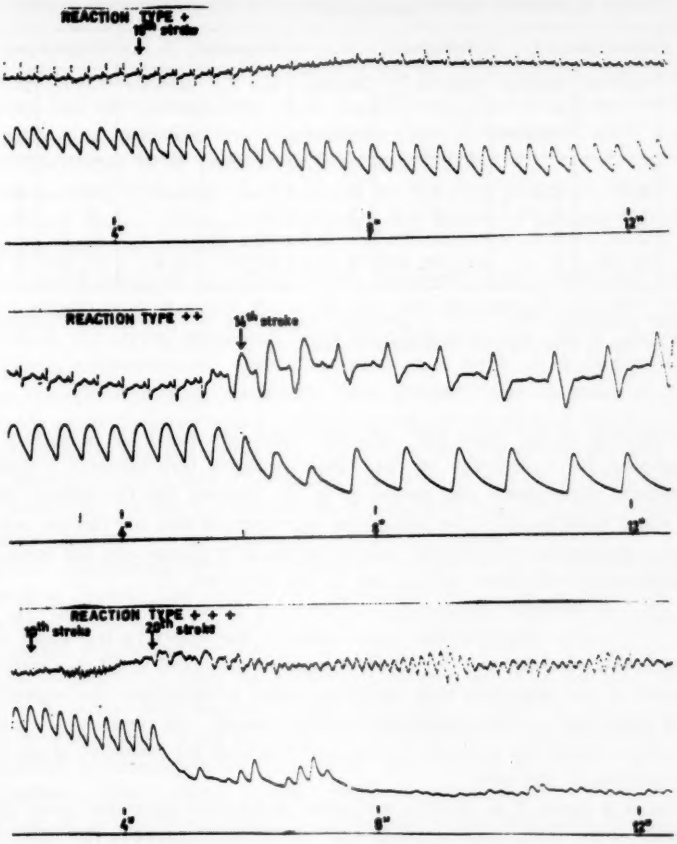

Fig. 2. Types of reaction to acute concentrated potassium injection. Type + = mild potassium effect, Type ++ = potassium block, Type +++ = potassium fibrillation.

changes as those described previously by others (*e. g.* WIGGERS *et al.* 1930, NAHUM and HOFF 1939). These changes have been characterized by lowering of the ST-interval, disappearance or negativity of the T-wave (Fig. 2. Reaction type +): The condition improwed in few seconds or developed into a block (Fig. 2. Reaction type ++), or was followed by potassium fibrillation (Fig. 2. Reaction type +++). The block could improve spontaneously in few seconds but the fibrillation could persist over a period of more than two minutes and then spontaneously improve. A high positive T-wave appeared 20—30 sec after the potassium injection or about 10—15 sec after the cessation of the fibrillation.

Table I. Reaction to potassium injections in atmosphere and in different respiratory disorders

Degree of reaction	Atmosphere		Asphyxia		Hypoxia		Hypercapnia	
	Cat no.	Number of injections	Cat no.	Number of injections	Cat no.	Number of injections	Cat no.	Number of injections
+	4, 9	2, 2	9	2	9	2	22	3
++	5, 6	2, 2	5	2	—	—	10	2
+++	8, 10, 11	8, 2, 1	4, 6, 7*	1, 1, 2	18*, 19*	1, 1	11, 16	3, 1
	12, 13	3, 3	11, 14*, 15*	2, 1, 1	20, 21	1, 3	17, 23*	5, 1

+ = mild potassium effect, ++ = potassium block, +++ = Potassium fibrillation as depicted in fig. 2. Cats marked with asterics died

The slowing of the pulse rate and the block provoked by potassium was accompanied by a decrease in the blood pressure but effective pulsations were present throughout this period (Fig. 2). During the fibrillation period on the other hand no effective pulsations were present and the electric activity of regular appearance which may appear at the later phases was not associated with mechanical efficiency and a rise in the pressure.

The effect of potassium injection in our series is presented in Table II. The results of the first experimental trial illustrate the effect in the cases when potassium was administered during the disorders produced at 30 min intervals. The order of the injections was varied in order to eliminate the cumulative effect of potassium on the interpretation of the results. The potassium injections were in each condition given in 5 min intervals and the intensity of the reaction is marked in the table.

In the first series 2 or more consecutive potassium injections were given. The effect of the injection is marked with +, if the changes in the ECG were moderate, ++ if they were followed by a block and +++ if a fibrillation was produced (vide Fig. 2). In the latter experimental trials attempts were made to produce such fibrillation and the number of injections necessary for this was reported.

The results in the table indicate that under normal atmospheric conditions the cats have tolerated the administered potassium distinctly better than in the disturbancy cases. Cats no. 4, 5, 9, 10 and 11 received injections first in asphyxia, hypoxia or hypercapnia and tolerated the injections in atmosphere well. When the injection was given in atmosphere first as in cat 6, the cat reacted very seriously when the injection was repeated in asphyxia. In hypercapnia the tolerance appeared better than in asphyxia or hypoxia. Respiratory acidosis appearcd therefore not to be a determining factor for the lowered potassium tolerance but that the role of hypoxia seems to be more decisive.

Discussion

The toxic effect of potassium in cats appears to be increased during oxygen lack. The results obtained in the experiments have somewhat varied in different animals but the observations made in different conditions have shown that the lack of oxygen has more importance than the excess of carbon dioxide (*e. g.* MATHISON 1910, GREENE and GILBERT 1922).

Permanent fibrillation was produced by the first potassium injection only in one cat during hypercapnia although the degree of acidosis was as great as in the asphyxia experiments.

The total amounts of administered potassium were small and obviously the applied concentrations have caused the changes. Even after repeated injections the serum potassium concentrations were not high. Likewise high potassium concentrations were not found as a result of respiratory acidosis even though occasional elevations were present. The response of the cats towards asphyxia seems thus to differ somewhat from that of the dog. Oxygen administration did not cause any cardiovascular changes in the cats. In dog the same has even caused a cardiac arrest (MILLER *et al.* 1952). Within normal pH conditions the dog has not tolerated potassium as well as during respiratory acidosis (SCRIBNER *et al.* 1954). In the cat the matter is reversed.

These experiments do not yet indicate the cause of the diminished potassium tolerance. Several factors may be responsible for it. The applied disturbancy of a duration of half of an hour has not been able to cause a consistent rise in the serum potassium concentrations. There may still be a tendency for an escape of potassium from the cells perhaps as a result of the lack of energy due to hypoxia. Also increased adrenal cortical activity at least in asphyxia may be responsible for the fact that the animals have not tolerated the applied exogenous potassium during respiratory disturbancy as well as in a normal atmosphere. Also a direct cardiac effect of potassium should be considered. The use of radioactive potassium — though — has not been able to clarify why some animals have recovered more rapidly from the potassium influence than others in studies by KAPLAN and FISHER (1959). Still the question is a matter of interest since potassium is used as a cardioplegic substance in open heart surgery (MELROSE *et al.* 1955). Obviously its use is accompanied by difficulties. The possible cause of them, we hope, have been partially elucidated by the present investigation.

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Acta physiol. scand. 1961. 52. 185—187

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The Effect of Histamine on the Uptake of ^{35}S by the Duodenal Mucosa of Guinea Pigs

By

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Abstract

Ball, P., I. Häkkinen and K. Hartiala. *The effect of histamine on the upptake of ^{35}S by the duodenal mucosa of guinea pigs.* Acta physiol. scand. 1961. *52.* 185—187. — The purpose of the work was to find out to what degree the effect of promoting the uptake of ^{35}S was dependent upon free access of acid to the duodenal lumen. The pylorus was ligated in 32 young male guinea pigs. Histamine in a dose of 3 mg/kg body weight was administered subcutaneously as the ^{35}S sulphate given immediately after the operation. The animals were sacrified 24 hours later. It was found that the uptake of ^{35}S sulphate was increased both in the duodenal mucosa and in the underlying "stroma". The results would indicate that in the guinea pig histamine stimulates the synthesis of mucus in the duodenum also under conditions where the effect of acid has been eliminated.

Brunner's glands secrete at a faster rate when acid is introduced into the duodenum (Florey, Wright and Jennings 1941). This response is at least partly mediated by the local release of a humoral agent (Sonnenschein, Grossman and Ivy 1947). Goblet cells empty themselves of mucus in response to local applications of acid or irritants; the factors which affect the rate at which they secrete are otherwise uncertain.

Häkkinen (1961) showed that in guinea pigs a single small dose of histamine would cause an increase in the quantity of sulphate released by acid hydrolysis from the deepest layers of the mucosa and the underlying connective tissue of the distal duodenum. It was concluded that the increase was dependent upon synthetic activity in mesenchyme. The increase could still be demonstrated after acid had been excluded from the duodenum by ligature of the pylorus.

Table I. The radioactivity of the tissues of the duodenal wall of guinea pigs 24 hours after injection of $Na_2{}^{35}SO_4$. Pyloric ligation was performed on all the animals. The treated group was also given histamine in beeswax 3 mg/kg body weight

Sample	Control group		Histamine treated group	
Counts/mg dry weight/min.	mean	S. E. M.	mean	S. E. M.
Duodenal mucosa.....	13.5	1.4	19.0	1.3
Duodenal stroma.....	5.2	0.6	9.9	0.7

In the superficial layers of the distal duodenal mucosa of intact animals the uptake of ^{35}S labelled sulphate was augmented by histamine, although histamine caused no perceptible change in its sulphate content as determined chemically. It was suggested that the uptake of labelled sulphate under these circumstances reflected the secretory activity of mucous cells.

The purpose of the work to be described in this paper was to find out to what degree the effect of histamine in promoting the uptake of ^{35}S labelled sulphate was dependent upon free access of acid to the duodenal lumen.

Material and methods

Thirty-two young male guinea pigs were used. The pylorus was tied under ether anesthesia. Immediately after operation ^{35}S was given subcutaneously as $Na_2{}^{35}SO_4$ (obtained from Radiochemical Centre, Amersham, England) in a dose of 400 μc/kg body weight, with 16 mg of Na_2SO_4 as carrier. The animals were then separated into two equal groups. One group served as controls. The animals in the other group were given histamine in beeswax subcutaneously in a dose of 3 mg/kg body weight in antihistamine cover (Antistine Ciba 20 mg/kg body weight subcutaneously). It was stated in preliminary experiments that antihistamine used had no effect on the sulphate content or uptake of the radiosulphate in the gastroduodenal tract of guinea pigs.

The animals were killed 24 hours after operation. The duodenum was prepared by the method described by HÄKKINEN (1961). In brief, the mucosa was scraped off from the underlying tissue of a standard length of duodenal wall, distal to the ampulla of Vater, in an area where in the guinea pigs BRUNNER's glands are diffuse and relatively scanty. Microscopical examination showed that the "mucosa" which was scraped away contained the villi and the greater part of the crypts of LIEBERKUHN, and that the "stroma" remaining consisted of some rests of the crypts, the muscularis mucosae containing BRUNNER's glands and the muscle and serosa. The dry weight of both mucosa and stroma were measured and the tissues were separately homogenised, hydrolysed and prepared for counting of radioactivity by a plate technique as previously described (HÄKKINEN 1961).

Results

The results are expressed as counts per minute/mg dry weight of tissue, and are shown in Table I.

The difference between the values for the two groups is significant for the mucosa ($0.01 > P > 0.001$), and highly significant for the stroma ($P < 0.001$).

Discussion

ODEBLAD and BOSTRÖM (1952) have shown that such radioactive sulphate as is fixed in the tissues is almost all incorporated into sulphomucopolysaccharides, and HÄKKINEN (1961) confirmed that in the duodenal wall nearly all suphate, whether determined chemically or in terms of radioactivity 24 hours after injection of ^{35}S, was contained in substances of high molecular weight. If on the other hand the radioactivity of the duodenal secretions is measured 4 hours after injection of ^{35}S sulphate or later, only some 69 % of the activity is found to be contained in high molecular organic compounds, that is in sulphomucopolysaccharides. The rest is present as inorganic sulphate or conjugated sulphate (KENT *et al.* 1956). This difference between the tissue and its secretions indicates that the duodenal mucous cells do not store inorganic or conjugated sulphate: the rate of transit of these substances through the duodenal wall is rapid, and the quantity present at any one time is accordingly very small.

The activity 24 hours after ^{35}S was given was the same in the untreated animals in this study as that recorded by HÄKKINEN (1961) in intact animals. The operation of pyloric ligation thus appears to exert no net effect. In the operated animals histamine caused an increase in activity of about 90 % in the deep layers of the duodenum. This change in the stroma as well as the increase in the sulphate content as measured chemically may well correspond to the suggested action of histamine on the synthetic activity of connective tissue. The activity increased about 40 % in the more superficial layers of the mucosa, whereas increase was about sixfold when there was normal continuity between the stomach and duodenum (HÄKKINEN 1961). If one accepts that the rate of uptake of ^{35}S sulphate into mucosal sulphomucopolysaccharides is proportional to the rate of synthesis of mucus in the gland cells, it is evident that histamine still exerted a stimulating effect upon this synthesis when acid had been excluded from the duodenum. Either the effect was directly produced by histamine, or some additional path way of stimulation exists whose receptors lie outside the duodenum.

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Acta physiol. scand. 1961. 52. 188—194

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Gastro-intestinal Excretion of Dextran-C^{14}

By

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Abstract

Åberg, B., W. L. Bloom and E. Hansson. *Gastrointestinal excretion of dextran-C^{14}*. Acta physiol. scand. 1961. *52*. 188—194. — Cats were given dextran-C^{14} intravenously and the gastric juice as well as intestinal juices from intestinal segments and bile was collected during the first 6 hours after administration. The juices were investigated for radioactivity. It was found that there is a small excretion of radioactivity *via* the stomach and the liver. The intestinal juices however and chiefly those from the cranial parts of the intestine contained larger amounts of radioactivity which was precipitable with ethanol and represented dextran. The earlier literature on dextran metabolism is reviewed. The parallelism between the dextran and the albumin elimination *via* the gastrointestinal tract is pointed out.

Partly hydrolyzed, bacterial dextran, introduced as a plasma substitute by Grönwall and Ingelman in 1944 has been extensively tried clinically during the last 15 years. The metabolism has been studied by numerous authors and their results were recently reviewed by *e. g.* Squire *et al.* (1955) and Grönwall (1957).

It has been demonstrated that starch-splitting enzymes do not break down dextran (Grönwall and Ingelman 1945, Ingelman 1947, Bull *et al.* 1949). Persson (1952) found evidences that dextran is altered in the tissues in a way which makes it stainable with leuco-fuchsin without previous treatment with periodic acid. This author presumed that the change was due to oxidation of dextran. Schmitz (1951) found that dextran was broken down by horse leucocytes *in vitro* as judged by viscosimetric determinations. Gray, Siiteri and Pulaski (1951) presented evidences that dextran is metabolized to glu-

cose in the phlorizinized dog. Uptake and storage of dextran in various organs *i. e.* liver, spleen, kidney, was reported by several investigators *e. g.* BULL *et al.* (1949), ENGSTRAND and ÅBERG (1950), FRIBERG, GRAF and ÅBERG (1951, 1953), SWEDIN and ÅBERG (1952).

Gastro-intestinal excretion of dextran was found by ENGSTRAND and ÅBERG (1950) in rats and rabbits and by TROELL and ÅBERG (1953) in man. Contradictory results were reported by several investigators (cit. GRÖNWALL 1957). The chemical methods employed for determination of dextran in biological fluids, have been and are very unspecific. Thus reactions with carbazol (ÅBERG 1952) or anthrone (DURHAM *et al.* 1950) will give results greatly influenced by all carbohydrates able to form furfural in the presence of conc. sulfuric acid. Turbidimetric determination after precipitation with ethanol as introduced by JACOBSSON and HANSEN (1952) gives fairly correct values in blood serum or plasma but cannot be used on gastro-intestinal contents. The nonspecificity of the chemical reaction might have caused the differences in opinion as to the gastrointestinal secretion of dextran.

BLOOM and WILHELMI (1952) presented evidence that there is an enzyme in the duodenal contents able to break down dextran rapidly and ÅBERG (1953) showed that human faeces breaks down dextran to low molecular fatty acids.

Synthesis of dextran-C^{14} made it possible to study the metabolism of dextran further. CARGILL and BRUNNER (1951) showed that dextran-C^{14} was metabolized in mice — the expired air of the animals contained $C^{14}O_2$ and the urine contained C^{14}-carbonate. In man, HELLMAN (1951) studied the metabolism of dextran-C^{14} (Commercial Solvents-Argonne National Laboratory Lot 21—2—Ci—L—D) and found that in 10 days 64 per cent of the radioactivity was excreted in the urine and 26 per cent as $C^{14}O_2$ in the expired air. About 2 per cent appeared in the faeces. HELLMAN did not study the gastro-intestinal contents of his patient.

Injection of tritium-labelled dextran into mice showed that there was a clear gastro-intestinal excretion of the tritium (HANNGREN *et al.* 1959). Although it was possible to precipitate the activity with ethanol from the urine which showed that the tritium had stayed on the dextran molecules it was deemed of interest to study the excretion further. A series of experiments with C^{14}-labelled dextran were performed on cats as one of us (W. L. B.) had dextran-C^{14} of the same brand as that used by HELLMAN (1951).

Material and Methods

C^{14}-Dextran

Generally C^{14}-labelled, partly hydrolysed dextran (Commercial Solvents-Argonne National Laboratory Lot 21—2—Ci—L—D) as a 6 per cent sterile solution in saline was used. The specific activity was 6 μC of C^{14} per g of dextran. Physical-chemical data

Table I. Excretion data for dextran C^{14}. (CSC-Argonne Lot no. 21-2-Ci-L-D)

Cat no.	Weight kg	ml of C^{14}-dextran	Cpm of C-14	Time (hrs after inj.)	Excretion as cpm in		
					Urine	Bile	Stomach
1	3.4	25	7.5×10^6	6	3.6×10^6	×	1.08×10^4
2	2.5	19	5.7×10^6	6	2.3×10^6	1.67×10^4	×
3	3.9	28	8.4×10^6	6	4.3×10^6	×	×
4	3.9	22	6.6×10^6	6	3.5×10^6	1.31×10^4	×
5	3.4	25	7.5×10^6	2	2.8×10^6	×	×
6	2.5	18	5.4×10^6	6	2.9×10^6	×	×
7	2.9	28	8.4×10^6	6	4.3×10^6	×	5.62×10^4

for this lot of dextran have been presented by WEISSBERG and ISBELL (1951) — light scattering revealed a mean molecular weight of 66.000.

Injection. The dextran-C^{14} was given in the femoral vein.

Urine samples: a polyethylene cannula was introduced via the urethra into the urinary bladder and urine samples were taken at 1 hour, 2 hours, 4 hours and 6 hours after the injection of dextran.

Blood samples: a permanent cannula was introduced into the carotid artery and blood samples taken 5 min, 15 min, 1 hour, 2 hours, 4 hours and 6 hours after injection of dextran.

Bile: A polythene tube was introduced into the hepatic duct after ligation of the cystic duct. The bile samples were taken at 1 hour, 2 hours, 4 hours and 6 hours after the injection of dextran.

Perfusion of the intestine. Cats starved 24 hours were anesthetized with chloralose urethane. An incision was made in the abdomen and a section was removed about 15 cm below the stomach and in some experiments 15 cm above the caecum. An incision was made in the gut and two rubber balloons was introduced into the intestine according to the arrangement described by ARMSTRONG, MARGEN and TARVER (1960). With this arrangement of balloons an about 20 cm long section of the intestine was isolated. The intestine was washed by flowing a 2 per cent dextran solution through a polyvinyl tube and taking out the perfusion fluid via another. The samples were taken at 1 hour, 2 hours, 4 hours and 6 hours after the injection of dextran. The washing solution was tested for blood by the benzidine method.

Stomach content. The oesophagus was ligated just above the cardia. A small incision was made in the duodenum and a glass cannula introduced into the stomach. The stomach secretion was stimulated by a subcutaneous injection of 0.1 mg histamine hydrochloride per 10 kg body weight every hour. The stomach content was removed by continuous suction and washing of the stomach with physiological saline. The samples were taken at 1 hour, 2 hours, 4 hours and 6 hours after injection of radioactive dextran.

Radioactive assay: The radioactivity in the samples of urine, blood, bile stomach and intestinal contents was estimated in a gas flow counter after plating an amount of the samples on aluminium dishes in infinitely thin layers according to RYDBERG (1958).

Assay for radioactivity in the ethanol precipitable fraction of the intestine contents and the stomach contents were carried out as follows: to a sample of the washing solution an equal amount of an ice-cold 20 per cent trichloroacetic acid solution was added

Excretion as cpm in		Excretion as per cent of dose in				
Ileum	Jejunum	Urine	Bile	Stomach	Ileum	Jejunum
×	×	48.0	×	0.14	×	×
×	×	40.4	0.3	×	×	×
×	1.27×10^5	51.2	×	×	×	1.5
×	×	53.0	0.2	×	×	×
7.8×10^3	×	37.3	×	×	0.1	×
3.23×10^4	×	53.7	×	×	0.6	×
×	1.36×10^5	51.2	×	0.7	×	1.6

in order to precipitate the protein and followed by centrifugation at 3,000 *g* for 10 minutes. The supernatant was taken and ethanol was added to a concentration of 60 % following centrifugation of 3,000 *g* for 10 minutes and the sediment was washed three times in ethanol. Thereafter the sediment was dissolved in water and plated on aluminium dishes in infinitely thin layers. The radioactivity was estimated in a gas-flow counter.

Results and Discussion

The individual results are summarized in Table I. Obviously there is an excretion of radioactivity in the stomach, the bile and the intestines. In the urine about 50 per cent of the dose was excreted within 6 hours after injection (Fig. 1). In Fig. 2 the blood plasma content of radioactivity — representing circulating dextran-C^{14} is given for 3 animals.

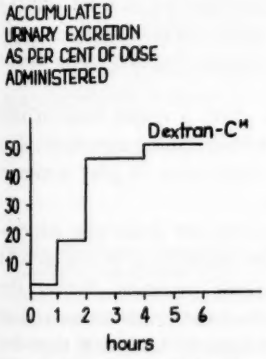

Fig. 1

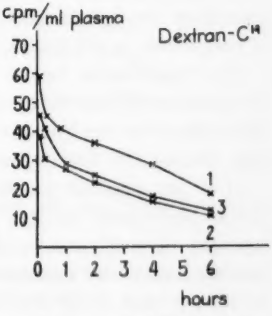

Fig. 2

Fig. 1. Cumulative elimination of radioactive material in urine of cat no. 3 following intravenous injection of dextran-C^{14}.

Fig. 2. Variation with time in blood plasma radioactivity following intravenous injection of dextran-C^{14} in cat no. 1, 2 and 3.

Table II. Percentage of radioactivity excreted precipitable with ethanol (60 per cent final concentration)

Cat no.	Juice/organ	Per cent precipitated
1	gastric	24.2
2	bile	18.9
3	jejunum	39.8
4	bile	72.2
5	ileum	82.9
6	ileum	59.0
7	jejunum	28.3
7	gastric	24.7

In the gastro-intestinal tract, the bile secretion of radioactivity is fairly small, amounting to some 0.2 per cent of the dose. The gastric secretion of radioactivity is also fairly small whereas the intestinal secretion is considerable, amounting to a maximum of 1.6 per cent of the dose in cat no. 7 in a 20 cm part of jejunum after 6 hours.

Of interest is the percentage of the radioactivity excreted which is precipitable with ethanol. Table II shows that this amount varies considerably. The variation at a final ethanol concentration of 60 per cent, is probably due to the bacterial and enzymatic decomposition of dextran which occurs in the juices during the collection time.

The urinary excretion (Fig. 1) and the plasma contents of radioactivity (Fig. 2) follow those earlier reported for this lot of dextran in *e. g.* man (HELLMAN 1951). The bile excretion is shown in Fig. 3 and is fairly small — as is seen from the figures given in Table I, the bile excretion has the same order of magnitude as the gastric excretion.

The experiments reveal that intestinal excretion plays a major role in the elimination of dextran intravenously administered to cats. Some excretion also takes place via the bile and the stomach but these routes seem to play a minor role during the first 6 hours after administration.

It is not possible to make any quantitative conclusions from the experiments performed but it is possible to make a rough estimation of the actual amount of radioactivity which appears in the whole intestine during the first six hours after injection of dextran-C^{14}. In cat no. 3 there was an excretion of 1.5 per cent of the dose in the 20 cm of intestine ligated. Assuming that the excretion is roughly the same in all parts of the intestine and that it has a total length of 1.4 m, about 10 per cent of the injected dose should be eliminated in this way.

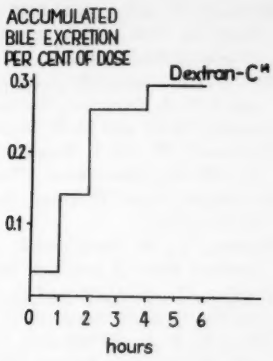

Fig. 3. Cumulative elimination of radioactive material in bile of cat. no. 2 following intravenous injection of dextran-C^{14}.

It can be concluded from these experiments that the intestinal elimination of dextran plays a major role in the cat. It has to be borne in mind, however, that the caudal part of the intestine eliminates far less dextran than the cranial part (vide Table I cats no. 5 and 6) and thus the actual amount of dextran excreted intestinally during the first 6 hours after *i. v.* administration must be far lower than 10 per cent. Even so intestinal excretion of dextran as well as urinary, must be considered the natural way of elimination of dextran. It is possible that serum albumin and perhaps other plasma proteins like dextran are excreted into the gastrointestinal tract where they are hydrolysed, since it has been showed that albumin-I^{131} is excreted in the stomach and small intestine by man and animals (Birke *et al.* 1958, 1959, Armstrong, Margen and Tarver 1960). Ullberg *et al.* (1960) showed autoradiographically that albumin-I^{131} is gastrointestinally excreted also in the cat. In "exudative gastro-enteropathy" a gastro-intestinal excretion of albumin has been presumed likely (Kimbel *et al.* 1956, Schwartz and Jarnum 1959 cit. Ullberg *et al.* 1960). These results suggest that gastrointestinal excretion of macro molecules may represent a hitherto unemphasized pathway of metabolism.

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Methods for Perfusing the Giant Axon of Loligo Pealii

By

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It was possible to perfuse the interior of the giant axon of *Loligo pealii* or *forbesi*, [C. S. Spyropoulos, I. Tasaki and T. Teorell, National Institutes of Health Annual Report, NINDB, 1959; Baker, P. F., A. L. Hodgkin and T. D. Shaw, Preprint of Proc. Physiol. Soc. (1961); also cited in preprint, Baker, P. F. and T. D. Shaw (1961), Plymouth Marine Biological Laboratory report for 1960—1961 (in press)] with an artificial solution and maintain the resting potential and the ability of the axon to produce normal action potentials. The technique employed (Diagram A) in the first series of experiments was as follows: Using an axon of *Loligo pealii* about 25—35 mm in length, a column of axoplasm was removed by introducing a capillary (300 μ in diameter). Subsequently each end of the axon was cannulated using glass pipettes (approx. 200—300 μ in diameter) and fine nylon thread. The perfusing fluid was introduced through one cannula and drained through the other. The rate of perfusion varied; in most experiments it was of the order of 0.01 ml/sec. The perfusion rate was usually but not always controlled with the Phipps and Bird Syringe-Driver. This rapid rate effected a rapid equilibration of the diffusible components of the intracellular space. A pair of silver wires was introduced through the cannula for stimulation and recording (under space clamp conditions). This electrode assembly facilitated drainage by guiding the perfusate from the ejecting to the withdrawing cannula. In another series of experiments, this was accomplished by means of a calomel micropipette which was also used as a potential recording electrode. In later experiments, simpler methods were used and longer axons (35—50 mm). In the method illustrated by diagram B a long glass capillary (350 μ in diameter) with a long slit (20 mm long and 100 μ in width) on one side was employed.

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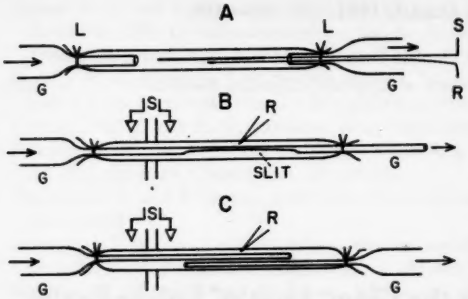

Fig. 1. *Illustration of various methods of perfusing isolated squid giant axons,* — A: Two separate glass cannulae (G) are tied with nylon thread (L) to the ends of the axon and a set of intracellular metal wire electrodes, for stimulation (S) and recording (R), are introduced through the distal cannula; when an accurate (D.C.) potential measurement is required, a calomel electrode is used. In this instance, external stimulatio is employed. B: A single glass c pillary provided with a long slit on one side is inserted to the extent that the slit is approximately half way through the axon and the tip protrudes through the end; external stimulation (S) across a vaseline gap and internal recording by means of a microelectrode pushed through the membrane are employed. C: Two separate cannulae, overlapping for about 20 mm in the middle portion of an axon, are used in conjunction with a microelectrode and external stimulating arrangements; the perfusate flows out of one cannula and is drained by the other. (Drawing not to scale.)

In C the perfusate was ejected through the opening of one of two overlapping (by about 20 mm) pipettes and collected through the opening of the second pipette. In another method, not illustrated in the figure, the perfusate flowed back toward the opening of the axon through which the perfusing glass capillary was introduced. In our technique there was always at the beginning a layer of axoplasm (50 μ or more) between the membrane and the zone of flowing fluid. Continuous perfusion usually resulted in erosion of the axoplasmic wall and in an appreciable reduction in its thickness.

Using isotonic KCl as a perfusate, the action potential remained normal for a period of approximately 30 min. This finding appeared to indicate that the internal sodium concentration could be reduced without changing the amplitude of the action potential. Conduction block was usually (but not always) preceded by prolongation of the duration of the action potential. Upon perfusing with sodium salt, instead of potassium, a rapid fall ensued in the resting potential and in the action potential amplitude. Using solutions of the same salt (Na or K) and fixing the concentration ratio across the membrane (with little adhering axoplasm), the membrane potential was found to depend on the level of the concentration ratios. Though the experiments were preliminary, this finding was interpreted as indicating the existence of fixed charge in the membrane (TEORELL, Progr. in Biophys. and Biophys. Chem., *3:* 305, 1953). The axons employed were 400—600 μ in diameter.

Quite recently, BAKER, HODGKIN and SHAW (cf. above reference) reported that they could remove nearly all of the axoplasm in their perfusion experiments. We cannot with certainty point to a particular difference in respective techniques that could account for their being more successful in maintaining the excitability for longer periods of time.